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Attenuation of the systemic inflammatory response syndrome following cardiac surgery using a pre-operative infusion of omega-3 fatty acids

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MD Thesis

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A thesis presented for the award of MD to the Royal College of Surgeons in Ireland, 123 St Stephen's Green, Dublin 2, based on research conducted at the Department of Surgery, RCSI Education and Research Centre, Beaumont Hospital, Dublin 9.

March 2010

Supervisors: Professor JM Redmond
Dr Jonathan McGuinness

Contents

Declaration

I declare that this thesis, which I submit to RCSI for examination in consideration of the award of a higher degree, Doctor of Medicine (MD), is my own personal effort. Where any of the content presented is the result of input or data from a related collaborative research programme this is duly acknowledged in the text such that it is possible to ascertain how much of the work is my own. I have not already obtained a degree in RCSI or elsewhere on the basis of this work. Furthermore, I took reasonable care to ensure that the work is original, and, to the best of my knowledge, does not breach copyright law, and has not been taken from other sources except where such work has been cited and acknowledged within the text.

Signed Niamh Keane

Student Number _____

Date 1/4/10

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Most importantly, I would like to thank my family and friends for their support and encouragement throughout these two years.

Abbreviations

IL-6 = Interleukin 6

IL-8 = Interleukin 8

IL-10 = Interleukin 10

TNF-alpha = Tumour necrosis factor alpha

ICAM-1 = Intercellular adhesion molecule 1

C3a = Complement factor 3a

GPIIb = Glycoprotein II b receptor

NFkB = Nuclear factor kappa B

mRNA = Messenger ribonucleic acid

SIRS = Systemic inflammatory response syndrome

ARDS = Acute respiratory distress syndrome

CABG = Coronary artery bypass graft

DHA = Docosahexanoeic acid

EPA = Eicosapentanoeic acid

AA = Arachidonic acid

PTCA = Percutaneous tranluminal coronary angioplasty

PGE3 = Prostaglandin E3

LTB4 = Leukotriene B4

IFN gamma = Interferon gamma

HSP = Heat shock protein

TPN = Total parenteral nutrition

NIRS = Near infrared spectroscopy

CVP = Central venous pressure

FiO_2 = Fraction of inspired oxygen

PEEP = Positive end expiratory pressure

ACT = Activated clotting time

MABP = Mean arterial blood pressure

pO_2 = Partial pressure of oxygen

pCO2 = Partial pressure of carbon dioxide

DHCA = Deep hypothermic circulatory arrest

LVEDP = Left ventricular end diastolic pressure

LVESP = Left ventricular end systolic pressure

CrCl = Creatinine clearance

GFR = Glomerular filtration rate

ARF = Acute renal failure

WCC = White cell count

LCOS = Low cardiac output syndrome

H&E = Hematoxylin and eosin

MPO = Myeloperoxidase

Summary

Advances in paediatric cardiac surgical techniques over the past two decades have resulted in increasingly successful complex corrective procedures, which are frequently performed on younger, more vulnerable infants. A degree of cardiac, pulmonary, renal and cerebral dysfunction is frequently seen post-operatively; this ranges in severity, but can in some progress to multiple organ failure. The exact mechanisms, pattern and timing of the post operative organ dysfunction following paediatric cardiac surgery has not been fully elucidated, however it is now appreciated that the systemic inflammatory response syndrome, induced by a number of factors present in cardiac surgery, has a central role. To date, much research has been undertaken in developing strategies to attenuate the SIRS; however, results are conflicting and none have consistently shown a benefit in clinical practice.

Omega-3 fatty acids have been shown to have anti-inflammatory, anti-infarct and anti-arrhythmic properties. Multiple pathways for these effects have been recognized: a reduction in pro-inflammatory cytokines and an increase in anti-inflammatory cytokines; an increase in the omega-3/omega-6 fatty acid ratio in cell membranes; an attenuation of the period of post-operative immunosuppression; and the production of resolvins and protectins, important in the resolution of inflammation.

The aim of this research therefore was two-fold: to determine the mechanisms involved in the injury induced by cardiac surgery, and to determine if omega-3 fatty acids, given in a clinically approved formulation, could attenuate the SIRS and produce beneficial

clinical effects in a juvenile piglet model of cardiopulmonary bypass and circulatory arrest.

The results obtained demonstrated a pattern of cardiac and pulmonary injury attributable to the SIRS. Renal injury occurred earlier and was not associated with SIRS. Omega-3 pre-treatment resulted in an attenuation of the systemic inflammatory response, as measured by cytokines and eicosanoids, and did demonstrate trends towards improved cardiopulmonary function.

This research provides a basis for further study into the mechanisms of post cardiac surgical organ injury, and also the attenuation of the SIRS with omega-3 pretreatment in the clinical arena.

CHAPTER 1

GENERAL INTRODUCTION:

1.1. Outcomes in paediatric cardiac surgery

Advances in paediatric cardiac surgical techniques over the past two decades have resulted in increasingly complex procedures focused on primary correction of the anatomical defects in many cases, or more complex palliation. These procedures often necessitate the use of deep hypothermic circulatory arrest or low flow cardiopulmonary bypass, such as the Norwood procedure, the first in a three stage repair of hypoplastic left heart syndrome, and the arterial switch operation for transposition of the great arteries. Others require prolonged cardiopulmonary bypass times, such as the repair of Tetralogy of Fallot or truncus arteriosus repair. In addition, these procedures are now frequently being carried out on younger, more vulnerable infants. Although overall mortality for congenital cardiac surgery has decreased[1], morbidity, particularly neurological, remains significant.

Cardiac surgery results in both ischemia-reperfusion injury and the induction of the systemic inflammatory response. Usually this response is mild or subclinical. However, in the current era of complex procedures in a patient population with reduced physiological reserve, both adult and paediatric, the response is often more severe and can result in multiple organ dysfunction post-operatively.

1.2 The systemic inflammatory response to cardiac surgery

Cardiac surgery induces the systemic inflammatory response via three main mechanisms: ischemia-reperfusion injury to the heart; gut ischemia; and contact with the cardiopulmonary bypass circuit[2]. Other mechanisms of activation of SIRS in cardiac surgery include complement activation by the heparin-protamine complexes[3] formed on reversal of heparinisation with protamine; and, when used, deep hypothermic circulatory arrest itself contributes to the SIRS[4], through the mechanisms of organ ischemia-reperfusion and NF kappa B modulation, as discussed below.

Myocardial ischemia-reperfusion injury may be global, as in cardiopulmonary bypass with cardioplegic arrest, or regional, as in off-pump surgery. Ischemia results in the production of reactive oxygen free radicals, which induce the release of pro-inflammatory cytokines including IL-6, IL-8 and TNF-alpha, in a proportional response to the length of the period of ischemia[5]. Reperfusion of the ischemic tissue further augments the inflammatory response, by also inducing the release of inflammatory cytokines and chemokines, increasing expression of adhesion molecules on the endothelium, and recruiting and activating monocytes and neutrophils. Tissue factor, which initiates the extrinsic coagulation cascade, is also contributory to the ischemia-reperfusion injury through the generation of thrombin[6]. Thrombin has demonstrated a pro-inflammatory role independent of its role in fibrin production and deposition, inducing expression of adhesion molecules P-selectin, E-selectin and ICAM-1, attracting polymorphonuclear leucocytes, and activating cells to produce IL-1, IL-6 and IL-8[7].

Non-occlusive mesenteric ischemia occurs with cardiopulmonary bypass as a result of hemodilution and non-pulsatile flow, which causes the release of endogenous splanchnic vasoconstrictors[8]. This results in a loss of the integrity of the tight intercellular junctions, thus increased intestinal permeability, and a leak of endotoxins into the systemic circulation. In addition, gut ischemia-reperfusion also induces the release of pro-inflammatory cytokines[9].

Contact with the bypass circuit causes activation of platelets and of the complement, kinin-kallikrein, and coagulation cascades.

Complement is activated by both the classic and alternative pathways – the classic pathway through a byproduct of Factor XII activation and by antibodies to the heparin-protamine complexes; the alternative pathway through failure of the bypass circuit to inactivate C3b (a normal function of the endothelium)[10]. The cascade then results in the production of C3a and C5a which activate neutrophils, and the membrane attack complex C5b-9 which damages endothelial cells.

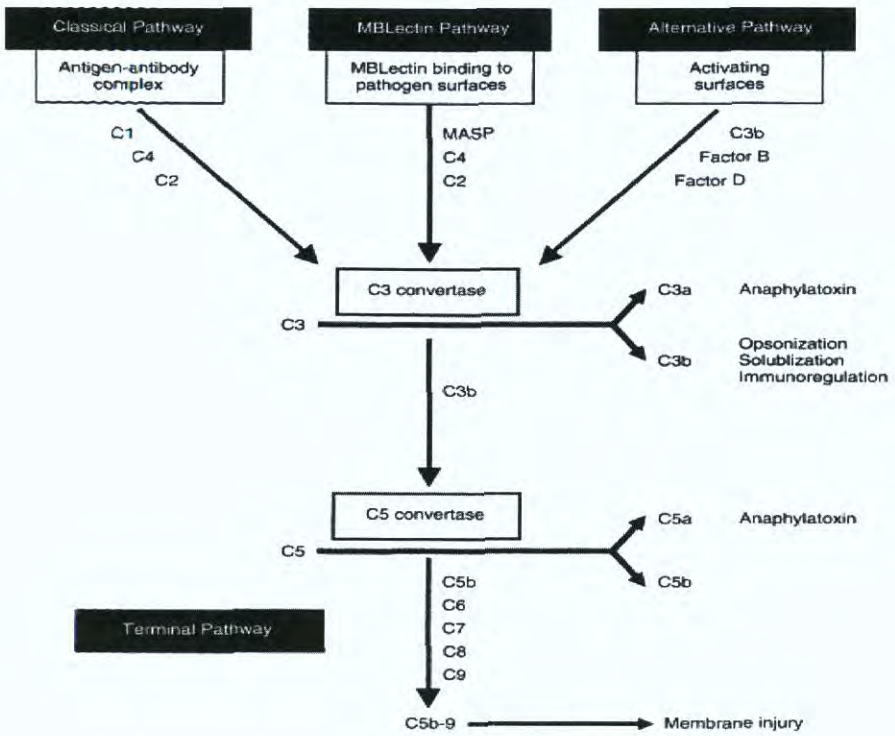


Figure 1: The complement cascade

Source: www.nature.com/ki/journal/v59/n4/images/4492147f1.gif

Plasma kallikrein is also activated through contact with the bypass circuit and results in the release of bradykinin, the production of plasmin and the activation of Factor XIIa, all of which cause further activation of neutrophils and the endothelium[2].

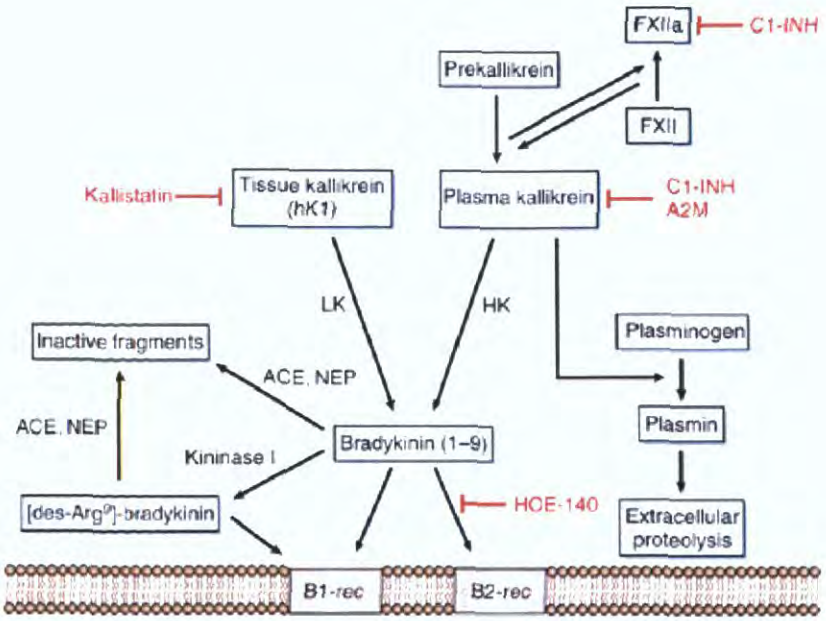


Figure 2: The kinin-kallikrein system

Source: www.nature.com/ki/journal/v73/n10/images/ki20089f1.jpg

The intrinsic pathway of the coagulation cascade is also activated by contact with the bypass circuit. This is also linked to the kinin-kallikrein and complement cascades through the common Factor XII. As mentioned earlier, tissue factor is generated in response to ischemia-reperfusion injury and thus activates the extrinsic coagulation pathway. Activation of the coagulation cascade can cause localized microthrombosis, thus resulting in further areas of ischemia[11].

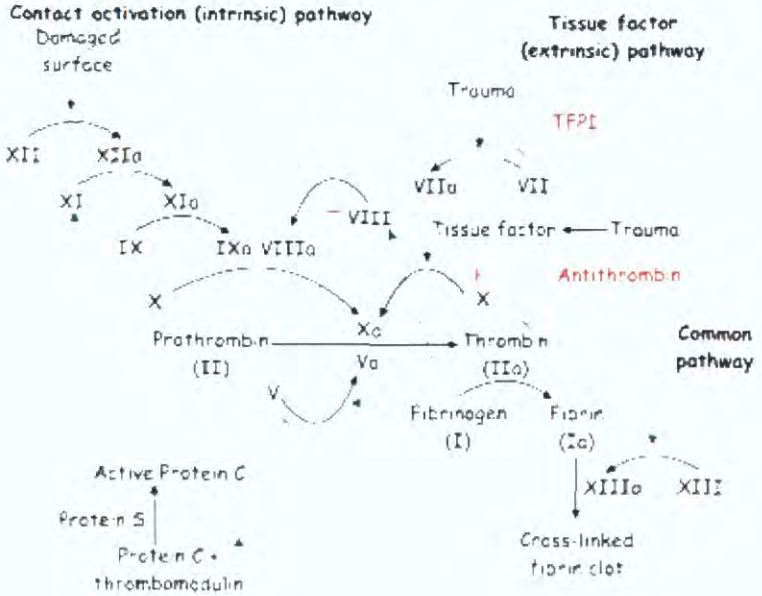


Figure 3: The coagulation cascade

Source: www.answers.com/topic/factor-xii

Plasmin, thrombin and fibrinogen produced as described above all have actions on platelets which combine to reduce both platelet numbers and efficacy. Plasmin activates platelets causing them to shed their GPIIb receptors – this results in reduced efficacy, but in addition, exposure to this receptor, which has been deposited in the bypass circuit, causes them to adhere, thus also reducing the number of circulating platelets[8,2]. Thrombin activates platelets leading to their aggregation, and fibrinogen deposited on the bypass circuit also activates and consumes platelets[8,2]. These effects on platelets have significant implications for post-operative hemostasis.

In summary therefore, the systemic inflammatory response is induced in the setting of cardiac surgery via three mechanisms – myocardial ischemia reperfusion injury; non-occlusive mesenteric ischemia; and activation of platelets and of the complement, kinin-kallikrein, and coagulation cascades through contact with the bypass circuit. Activation of circulating inflammatory cells and endothelial cells activates cellular transcription factors, most importantly nuclear factor kappa B (NFkB), which then results in transcription of mRNA and cytokine synthesis[12,13]. The inflammatory mediators thus produced result in the continued activation of the endothelium with further inflammatory cytokine release and upregulation of adhesion molecules. Circulating activated neutrophils, the main effectors of the inflammatory response, consequently adhere to the endothelium, and then transmigrate into the tissues where they release reactive oxygen species and damaging tissue enzymes such as myeloperoxidase, elastase and metalloproteinases. These leucocyte-endothelial interactions have been demonstrated to be the key pathological process in endotoxin[14] and ischemia-reperfusion injury[15].

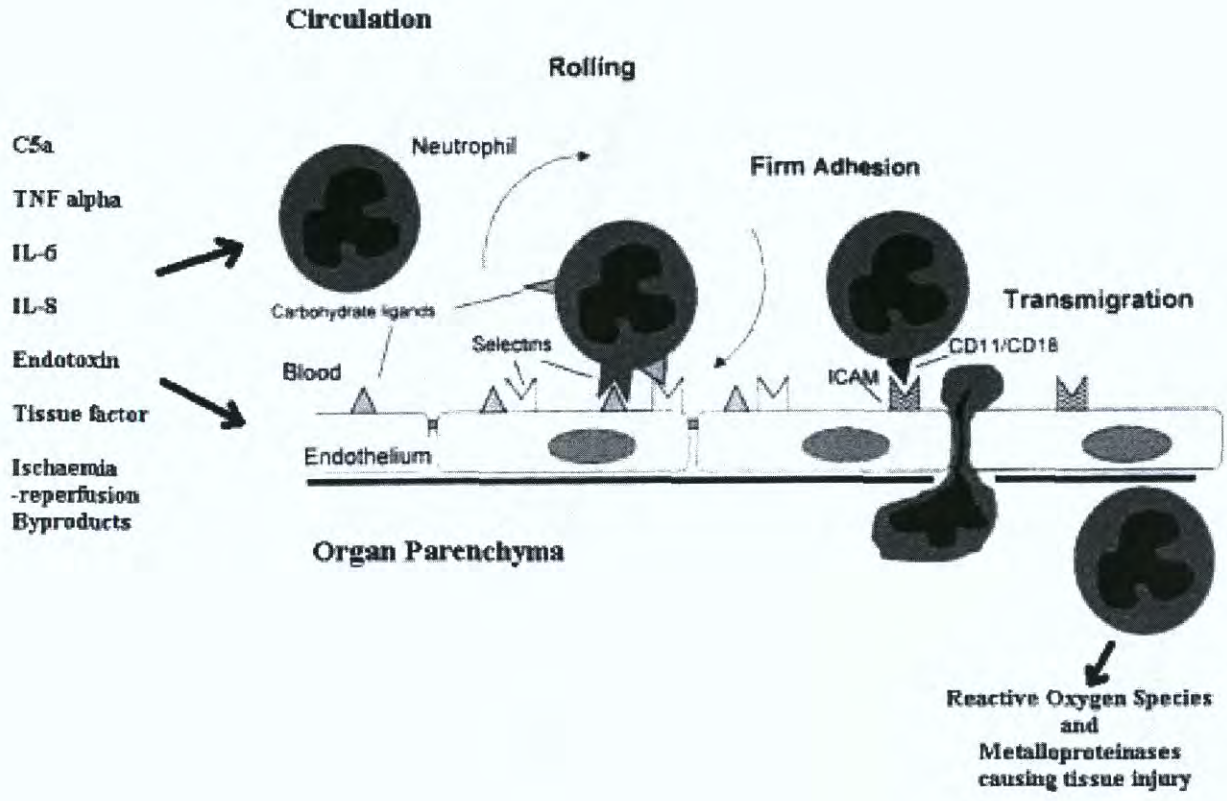


Figure 4: The stages of leucocyte endothelial interactions

Inflammatory mediators, such as IL-6, IL-8 and TNF-alpha, activate neutrophils and the endothelium. Initially selectins, which are expressed early on the endothelium, result in slowing and weak binding of the circulating neutrophils. Following this, integrins, expressed both on the endothelium and the neutrophils result in firm binding of the neutrophils to the endothelium, and primes them for degranulation. Transmigration of the neutrophils into the tissue is the next step, followed by the release of reactive oxygen species and tissue damaging enzymes. In a normal inflammatory response to injury, this response is limited to an infected or

damaged area of tissue; however, in the inflammatory response to cardiac surgery, the endothelial activation is widespread, therefore so too is the damage.

The effects of the SIRS are maximal within the first 24 hours post operatively[2], therefore this is the time period during which therapeutic interventions would be most beneficial. As the inducers of the inflammatory response, complement factors (C3a, C5a and the membrane attack complex C5b-9) and endotoxin are the first measurable mediators in the circulation[8,16]. The pro-inflammatory cytokines IL-6, IL-8 and TNF-alpha peak at approximately 2-4 hours post cardiopulmonary bypass; their activity leads to the activation of neutrophils and the endothelium, which is seen as an increase in the number of neutrophils, the neutrophil specific adhesion molecules CD11b, selectins and integrins between 3 and 12 hours. From 12 hours on, all of these pro-inflammatory mediators begin to decrease and are at almost baseline levels by 24 hours.

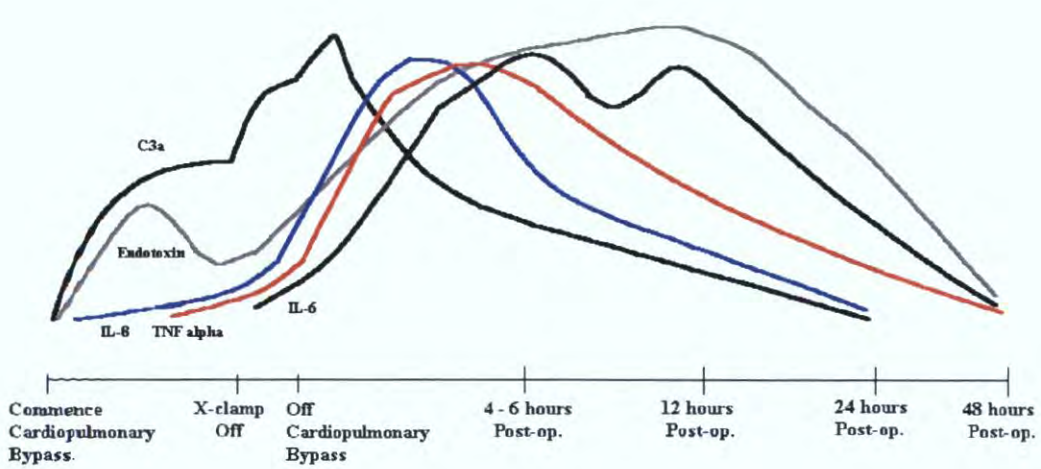


Figure 5: Time course of inflammatory mediator appearance in the circulation

Source: Surgeon 2008; 6(3):162-71

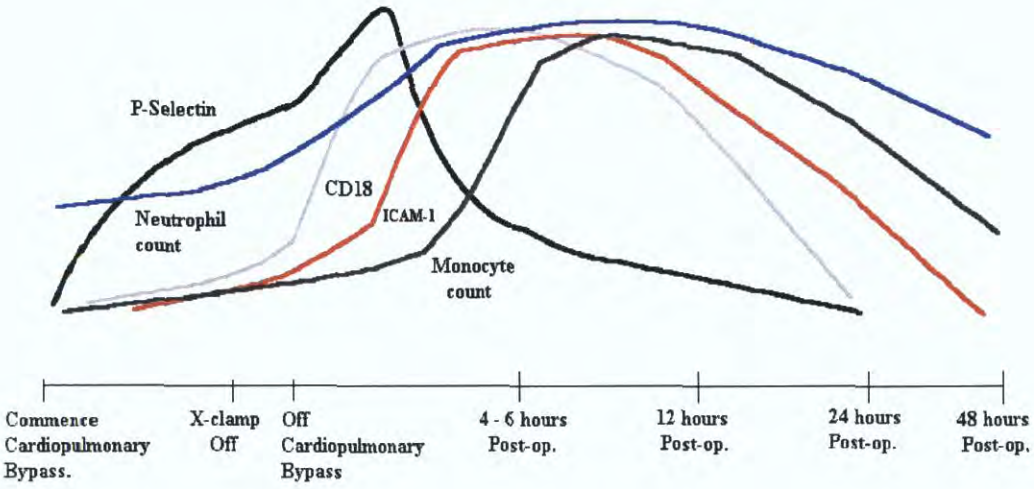


Figure 6: Time course of the leucocyte and adhesion molecule appearance in the circulation

Source: Surgeon 2008; 6(3):162-71

1.3 Clinical Implications

In the majority of cases, the clinical manifestations of the SIRS and ischemia-reperfusion injury post cardiac surgery are subclinical or mild. However, with the increasing frequency of cardiac surgery in the more vulnerable adult and more complicated prolonged procedures in the paediatric population, the potential for multiple organ dysfunction is increased, and therefore the need for safe therapeutic interventions more pressing. To date, numerous strategies have attempted to attenuate the SIRS and ischemia-reperfusion injury post cardiac surgery; the only one of which has been incorporated into standard clinical practice is cardioplegia. Other potential therapies include: leucocyte depletion[34,56,57], pulsatile cardiopulmonary bypass[17], heparin coated circuits[18], and N-acetylcysteine[47](reactive oxygen species scavenger). However, all of these therapies target only one factor in the activation of the post cardiac surgical SIRS, therefore the remaining unaffected factors continue to activate transcription factors such as NFkB with resultant inflammatory mediator production, leucocyte-endothelial interactions and tissue damage continuing.

1.3.1 Cardiac Injury

Myocardial dysfunction following cardiopulmonary bypass is frequently encountered in the post-operative period, with inotropic support sometimes necessary on discontinuing bypass and over the first 24 hours in particular. Both systolic and diastolic ventricular dysfunction can be observed, and this in conjunction with vasomotor disturbances leading to reduced peripheral vascular resistance, can lead to a low cardiac output syndrome[19].

Post operative arrhythmias are also common, and are generally considered benign transient events[20]. However, severe arrhythmias may be associated with serious complications such as hemodynamic compromise, and thromboembolic events. Post operative arrhythmias are thought to be multi factorial, however incomplete myocardial protection is a major cause[21].

The pathology of the cardiac injury seen is due to the deliberate period of ischemia followed by reperfusion and the systemic inflammatory response, both of which lead to inappropriate leucocyte-endothelial activation. Multiple studies have investigated the role of inflammatory mediators and leucocyte-endothelial interactions in cardiac dysfunction. High levels of endotoxin have been associated with reduced myocardial function post-operatively, while in an animal study, endotoxin removal using a polymixin B-immobilized hemoperfusion cartridge resulted in an improved rate of recovery of left ventricular contractility and cardiac output[22]. Plasma levels of IL-6 have been correlated with postoperative myocardial ischemic episodes and echocardiographic wall motion abnormalities in adults[23], and a recent study in a neonatal population demonstrated that IL-6 level 4 hours post operatively was an independent risk factor for myocardial dysfunction[24]. IL-8 and TNF-alpha have also been studied: IL-8 is positively correlated with the length of inotropic support[25]; while high TNF-alpha levels are associated with reduced left ventricular contractility[26]. With regard to leucocyte-endothelial interactions, myeloperoxidase staining and electron microscopy of the heart in a canine ischemia-reperfusion model demonstrated large numbers of neutrophils two hours after weaning from cardiopulmonary bypass[27]. Furthermore, a clinical study which measured

neutrophils, elastase and lactoferrin simultaneously in myocardial and peripheral venous blood just after aortic unclamping showed reduced neutrophils in the myocardial blood compared with peripheral blood, suggesting myocardial sequestration, and higher levels of elastase and lactoferrin in the myocardial blood, suggesting degranulation of the activated neutrophils in the myocardium[28]. In addition, inhibition of neutrophil CD11b/CD18 in a juvenile piglet model of ischemia-reperfusion, reduced neutrophil accumulation in the myocardium, reduced myocyte damage and improved ventricular systolic function[29].

Thus, with the central role of the SIRS and leucocyte endothelial interactions in the pathophysiology of post operative myocardial dysfunction, modulation of these responses with a safe clinically applicable intervention would be expected to produce significant clinical benefit.

1.3.2 Pulmonary Injury

Pulmonary dysfunction is common following cardiac surgery, as can be measured by reductions in compliance and alveolar-arterial oxygenation gradient, and in the presence of pulmonary oedema. The clinical significance of this injury varies, with the most severe form of injury, ARDS (acute respiratory distress syndrome), occurring in 0.5 – 1.7% of the adult population, with a mortality rate of up to 90%[30]. In the paediatric population, acute lung injury is similarly common, with infants frequently requiring prolonged mechanical ventilatory support: recent studies of outcomes following the Norwood

procedure and repair of tetralogy of Fallot showed a median length of ventilation of seven days[31,32].

Similar to the cardiac dysfunction, it is the ischemia-reperfusion injury to the lungs and the subsequent induction of the SIRS and leucocyte-endothelial reactions that are felt to be the pathological process predominantly mediating this complication. This may be because the lungs are the only organ perfused with the whole cardiac output, and are thus exposed to the high levels of inflammatory mediators produced by the myocardium (a major source of IL-6 and TNF-alpha)[33]. Several studies have demonstrated the association of the SIRS with acute lung injury, and that modulation of this response leads to an improvement in post operative respiratory function. High plasma C3a levels have been associated with a prolonged requirement for mechanical ventilatory support[34]; while in an animal model, treatment with an inhibitor of C3 and C5 resulted in a reduction of pulmonary vascular resistance[35]. A number of studies have demonstrated neutrophil sequestration in the lungs; while the importance of neutrophil activation has been shown by attenuation of lung injury using leukocyte depletion with an arterial filter[36], and by administration of pentoxifylline, an inhibitor of leukocyte activation[37]. Elastase, a tissue damaging enzyme released by activated neutrophils, has also been studied: a positive correlation between plasma levels and respiratory dysfunction has been shown[38]; and inhibition using ulinastatin attenuated this injury[39]. In the pediatric setting, both clinical and animal studies have demonstrated improved post operative pulmonary function with pre- and intra-operative steroid administration through a reduction in inflammatory mediators[40 41].

The majority of proposed therapeutic interventions are focused on one mediator of the inflammatory response, and while promising results have been seen in animal studies, these have not translated into reproducible definite clinical benefits in terms of mortality and morbidity, probably due to the small numbers in most studies and the low overall incidence of severe lung injury. However, the exact timing and aetiology of lung dysfunction still remains unclear, with early post-operative oedema often attributed to cardiogenic oedema, which may not be the case.

1.3.3 Renal Injury

Acute renal dysfunction post cardiac surgery is a significant source of morbidity and mortality, both in adult and paediatric populations. The incidence varies depending on the definitions used, but ranges between 1% and 30%, with 1 – 5% of patients requiring renal replacement therapy[42,43,44]. Associated mortality is high: a recent study by Bove et al of over 5000 patients undergoing cardiopulmonary bypass in a single institution reported a hospital mortality of 40.9% in patients with acute renal failure and 63.8% in those who required renal replacement therapy[45]. These figures are in line with several other studies in the literature[46,47]. A paediatric study by Skippen et al reported an 11% incidence of acute renal injury (defined as doubling of creatinine) and 1% of acute renal failure (tripling of creatinine); none of the 101 children studied required dialysis[48].

The pathophysiology of renal injury post cardiac surgery appears to be multifactorial, with ischemic; inflammatory; and nephrotoxic causes, such as antibiotics, analgesics,

contrast media, and diuretics, all contributing to acute tubular necrosis[49]. Pre-operative risk factors include: reduced left ventricular function or congestive cardiac failure; diabetes mellitus; elevated serum creatinine; use of an intra-aortic balloon pump or the need for emergency surgery. Many of these factors would be indicative of reduced baseline renal perfusion and a reduced functional reserve. Intra-operatively, maintenance of renal perfusion through adequate flow rates and perfusion pressure is of paramount importance. A study in a porcine model by O'Dwyer et al demonstrated that improved renal perfusion was achieved by increasing flow rates rather than vasoconstriction with phenylephrine to increase perfusion pressure[50]. Studies have demonstrated reduced rates of acute renal injury with off-pump cardiopulmonary bypass[51,52], which may be related to the avoidance of the cardiopulmonary bypass circuit and the inflammatory response thus induced, or with the maintenance of pulsatile flow which avoids the renal vasoconstriction, and thus regional hypoperfusion, seen with non-pulsatile flow[53,54]. In addition, hemodilution (hematocrit < 25%), thus reduced oxygen carrying capacity, during bypass has recently been shown to be a risk factor for renal injury[55]. Post operatively, any factor which reduces the perfusion to the kidney can further exacerbate renal injury – hemodynamic instability, volume depletion and the need for vasoactive medications – as can the use of nephrotoxic agents. The role of the induced systemic inflammatory response in renal injury has yet to be fully elucidated. High IL-6 levels have been associated with acute renal dysfunction post cardiac surgery[56]; however studies with steroid administration have shown that despite a reduction in the SIRS, there is no clinical protection against renal injury[57]. Leucocyte depletion using in line arterial filters likewise has shown conflicting results[58,59].

Several pharmacological interventions have attempted to protect against the renal injury seen following cardiac surgery, however to date none of these have produced consistent results. The pathophysiology is complex, and as yet, incompletely understood, therefore full elucidation of the mechanism of injury will be necessary in order to appropriately target this significant and serious complication.

1.3.4 Cerebral Injury

Neurological complications following cardiac surgery are of particular importance, both in terms of their frequency and their impact on the quality of life of patients. In the adult population, a study from 2001 of 261 patients undergoing CABG reported post-operative cognitive impairment in 53% of these patients at the time of discharge, 36% at six weeks and 24% at six months[60]. However, although these early changes are no longer evident at one year post cardiopulmonary bypass, concerns have been raised regarding a late decline 5 years post surgery[39,61]: for example, Newman et al reported an incidence of 42% at five years[39]. However more recently, studies of CABG patients (both on and off pump) vs non surgical controls (both with medically managed coronary artery disease and without coronary artery disease) looking at evidence of cognitive dysfunction at one and three years post cardiac surgery, found no difference between groups[62,63]. This can be explained by a number of factors: these patients are older and therefore at risk of cognitive decline from normal aging, vascular disease (there is significant overlap of coronary artery disease and cerebrovascular disease), and Alzheimher's disease. While it is reassuring that these early cognitive changes in adults are likely to be transient, they

are still highly significant in that they can delay extubation or lead to sternal wound complications as a result of disruptive behaviour, delay discharge from hospital and slow rehabilitation[39].

In the paediatric population, as mortality from congenital cardiac surgery has decreased significantly, increasing attention has now focused on reducing morbidity, particularly with regard to neurodevelopment. Within the first six months following surgery, signs of cerebral damage were found in 6.26% of 534 children; these included seizures, movement disorders, cerebral hemorrhage, infarction, hydrocephalus or marked atrophy[64]. A study by Hovels-Gurich et al from 2006 which assessed children at a mean age of 7.4 years after corrective cardiac surgery in infancy found neurological abnormalities in 33%, all considered mild; and marked developmental impairment when compared to normal children – this included gross motor dysfunction in 50%, reduced formal intelligence in 25.5%, and reduced expressive language ability in 34.4%[65]. A study by the same group also assessed children after neonatal arterial switch operation at a mean age of 10.5 years, and again reported neurological (27%) and developmental impairment (55% in one or more domains) compared to the normal population[66]. These neurological deficits have important consequences for the individual child and their family – a study by Williams et al of 106 children and adolescents utilizing the children's health questionnaires showed that hypoplastic left heart syndrome patients scored lower in the following areas: role/social limitations due to emotional or behavioural difficulty, behavioural, self-esteem, global health, emotional parental impact, family activities and the psychosocial summary score[67].

The aetiology of the observed neurological injury is predominantly due to an ischemic insult, mainly through cerebral microemboli and also reduced cerebral perfusion peri-operatively. Cerebral microemboli, both solid and gaseous, can be directly observed using transcranial Doppler monitoring, and increased numbers have been correlated with early neuropsychological deficits[68]. Intra-operative hypoperfusion occurs with cardiopulmonary bypass, particularly in the paediatric setting where a period of deep hypothermic circulatory arrest or low-flow cardiopulmonary bypass may be used. However, the systemic inflammatory response to cardiac surgery is felt to modulate the response to the ischemic injuries so generated[69]. A recent review article examining the factors contributing to neurological injury post cardiopulmonary bypass in neonates indicated that there is strong laboratory data to suggest a causal relationship between inflammation and post cardiopulmonary bypass neurological injury; however the clinical research in this area has yielded inconclusive results[70]. Research in the area of cerebral ischemia has demonstrated that deleterious leukocyte involvement is promoted by inflammatory mediators released after the onset of cerebral ischemia, and that these mediators have a central role in the progression from ischemia to cellular death and necrosis[71]. In attempting to prevent the adverse neurological outcomes following cardiac surgery, research has focused on improving cerebral perfusion (minimizing microemboli, selective cerebral perfusion[72] or low flow cardiopulmonary bypass) and improving neuronal protection (hypothermia and topical cooling, maintaining higher hematocrits). In terms of the inflammatory response, a study by Mathew et al showed that lower pre-operative endotoxin immunity in patients undergoing CABG predicted increased cognitive dysfunction[48]. Also an interesting study demonstrated reduced ischemic brain

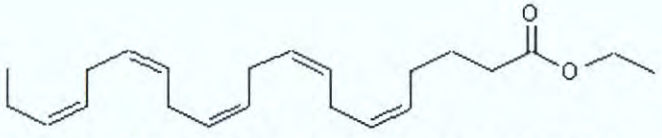
injury in interleukin-1 beta converting enzyme (ICE) knockout mice – ICE is necessary for the conversion of proIL-1beta to its biologically active form as a pro-inflammatory cytokine[73]. However, studies on the neuroprotective effects of steroids have produced conflicting results[74].

In summary, the neurological outcomes of cardiac surgery are of paramount importance, especially in paediatric surgery. This injury is multifactorial, and it is important therefore, in attempting to optimize management, to determine if the modulation of the SIR to cardiac surgery could improve post operative neurological outcomes.

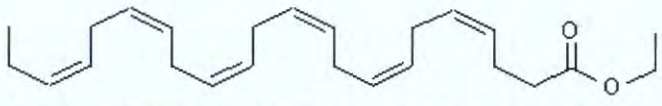
1.4 Omega-3 fatty acids

Fatty acids are derived from the hydrolysis of the ester linkages in triglycerides, and are either saturated or unsaturated: saturated contain only single carbon bonds; unsaturated contain double bonds, and may be mono- (one single bond) or poly-unsaturated (more than one bond). Mammals do not have the necessary synthetic mechanisms to introduce double bonds in fatty acids beyond carbons 9 and 10 in a chain, therefore the omega-3 and omega-6 fatty acids, whose first double bond is the third or sixth carbon-carbon bond, respectively, from the terminal methyl end of the chain, are considered essential fatty acids, meaning they must be obtained from the diet. The three most important omega-3 fatty acids are: alpha-linolenic acid (ALA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), which contain respectively 3, 5, or 6 double bonds in a carbon chain of 18, 20 or 22 carbon atoms. Although mammals cannot make omega-3

fatty acids de novo, EPA and DHA can be made from ALA. Arachidonic acid (AA) is the most important omega-6 fatty acid[75].



EPA: ethyl ester of eicosapentanoic acid



DHA: ethyl ester of docosahexaenoic acid

Figure 7: The molecular structures of eicosapentaneoic acid (EPA) and docosahexaenoic acid (DHA)

Source: www.eu-pharmgate.com

The benefits of omega-3 fatty acids were initially realized during the 1970s when epidemiological studies on Greenland Eskimos demonstrated very low rates of atherosclerotic heart disease in a population whose diet is naturally rich in omega-3 fatty acids. Interestingly, it was also found that arthritis and other chronic inflammatory diseases were almost unknown among this group. The Mediterranean diet is also naturally rich in omega-3 fatty acids and again epidemiological studies in this group have demonstrated reduced cardiovascular disease[76]. These observations prompted much research into the beneficial effects of omega-3 fatty acids and their mechanism of action. In cardiovascular disease, omega-3 fatty acids now have an important role both in the primary and secondary prevention of myocardial infarction. Three large trials reporting over a ten year period demonstrated highly significant reductions in sudden cardiac death

following a first myocardial infarction, ranging from 29% - 70%, with increasing dietary fish oils: in the DART trial[77] and Lyon Heart Study[78], this was achieved through dietary advice to increase fish oils; the GISSI Prevensaione trial used supplementation with EPA and DHA[79]. Primary prevention was examined in the Physicians Health Study[80] which demonstrated that increased blood levels of omega-3 fatty acids reduced the relative risk of sudden cardiovascular death by up to 81%. These results are felt to be due to the anti-arrhythmic properties of omega-3, and in vitro and in vivo work has demonstrated the mechanism of action to be due to incorporation of the long chain fatty acids into the cell membranes which produces stabilizing effects on ion channels[63]. This beneficial effect has been applied to CABG patients, where supplementation with omega-3 fatty acids peri-operatively reduced the incidence of post-operative atrial fibrillation by 54.4%[81]. Other benefits in cardiac patients include improved serum lipid profiles and the stabilization of atherosclerotic plaques[82 83]. Also, supplementation with omega-3 in patients post coronary artery bypass grafting demonstrated reduced vein graft occlusion at 1 year[84]; however studies examining restenosis rates following PTCA have yielded conflicting results[85].

Current clinical studies in surgical patients, including those undergoing major abdominal surgery (both oncological and non-oncological), trauma and critically ill septic patients, have repeatedly demonstrated beneficial anti-inflammatory effects with both enteral and parenteral supplementation with omega-3 fatty acids. Significant reductions in infectious complications, reduced intensive care and overall hospital stays, and fewer days of ventilation have all been demonstrated[86]. Bacterial translocation has been shown to be

reduced in an animal study[87]. Omega-3 fatty acids have also been shown in vitro and in vivo to inhibit the growth of cancer cells, and further clinical studies are investigating the potential benefits in this area[88].

The mechanisms of the observed anti-inflammatory benefits have been studied extensively. Fatty acids are incorporated into cell membranes as phospholipids. In response to an inflammatory stimulus, phospholipases cleave AA (omega-6) or EPA (omega-3) from the phospholipids and release them as free fatty acids. These are then converted through one of two pathways generating eicosanoids: the cyclo-oxygenase pathway produces the prostanoids; the lipoxygenase pathway produces the leukotrienes. The cell membrane ratio of omega-3 to omega-6 fatty acids is very important in modulating the immune response as the eicosanoids produced by the conversion of EPA are anti-inflammatory (prostanoids of the three series – thromboxane A_3, PGE_3, PGI_3; leukotrienes of the five series – LTB_5, LTC_5, LTD_5), while those derived from AA are pro-inflammatory (prostanoids of the 2 series, leukotrienes of the 4 series) and these two substrates will compete for conversion. Therefore, an increased ratio of omega-3 to omega-6 fatty acid in the membranes would be expected to produce anti-inflammatory effects[89].

Omega-3 also reduces the production of inflammatory cyokines, such as IL-6, IL-8, TNF-alpha, and IFN-gamma, and can decrease adhesion molecule expression leading to a reduction in leucocyte endothelial interactions, one of the main injurious pathways in the systemic inflammatory response. These effects may be due in part to the antagonism of

the production of the AA derived mediators, but are also likely to be due to direct actions on intracellular signaling pathways which lead to the down-regulation of transcription factors such as NFkB[90,91]. Recent interest in pre-conditioning, the phenomenon whereby a brief exposure to a non-lethal stimulus, such as ischemia, protects cells or tissues against a subsequent normally lethal stimulus, has shown that the protection afforded through this mechanism is due in part to the induction of heat shock proteins[92]. Previous studies in our laboratory demonstrated up-regulation of HSP72 following pre-treatment with omega-3 fatty acids, suggesting that this may be one of the mechanisms by which omega-3 protects cells and tissues[95,96].

In addition to its beneficial effects in reducing inflammation, omega-3 has also been shown to ameliorate the immunosuppression seen in patients post surgery, or in situations of trauma or sepsis. Increased CD4 lymphocyte production and an increased CD4/CD8 ratio have been demonstrated, as has improved phagocytic activity of macrophages, thus increasing the immunological cell response[93,94].

Beneficial anti-thrombotic properties of omega-3 have also been demonstrated, mediated through effects on platelets, through a reduction in thromboxane A2, and thrombomodulin. These effects are important both in terms of clot formation and in atherosclerotic plaque formation and progression.

Importantly, the omega-3 fatty acids are safe and well tolerated. Initially, concerns were raised regarding bleeding times, however further studies have shown fatty acids to be safe

in this regard[95]. Total parenteral nutrition regimes supplemented with omega-3 fatty acids have been administered in all settings, including the paediatric population[96], with minimal side effects reported.

1.5 Objectives

This research follows on from the work of Dr Jonathan McGuinness, PhD, and Dr John Byrne, MD, which demonstrated in vitro (cell work) and in-vivo (intravital microscopy) a reduction of leucocyte-endothelial interactions using Omegaven, a clinically acceptable TPN component consisting of omega-3 fatty acids[97]. A rabbit regional ischemia model also demonstrated a 40% reduction in myocardial infarct size with omega-3 pretreatment[98].

The specific aim of my research was to determine if these benefits seen with omega-3 pretreatment translated into multiple organ protection in a clinically relevant model of paediatric cardiac surgery. To this end, a juvenile piglet model of cardiopulmonary bypass and deep hypothermic circulatory arrest was developed in our laboratory, examining multiple organ function post-operatively. The aim was to determine the pattern, extent and timing of dysfunction in the heart, lung, kidneys, and brain; and to investigate the mechanism of injury so produced. I then aimed to determine if a pre-operative infusion of Omegaven attenuated this injury, and to investigate the mechanism of any protection afforded. This was achieved with an initial study comparing omega-3 pretreatment to controls over an eight hour period of post-operative observation, followed by a second study extending the period of observation to 24 hours. This work provides

the basis for a clinical study in the paediatric cardiac surgical population of omega-3 fatty acid pretreatment.

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CHAPTER 2

MATERIALS AND METHODS:

2.1.1. Juvenile Piglet Model of Cardiopulmonary Bypass and Deep Hypothermic Circulatory Arrest

2.1.1. Background

The juvenile piglet model of cardiopulmonary bypass and deep hypothermic circulatory arrest has been used extensively over the last two decades to assess the optimal physiological parameters that should be maintained peri-operatively in children undergoing cardiac surgery. It has also been used to assess the efficacy of proposed beneficial interventions, such as steroid pretreatment[1,2], intra-operative administration of *neutrophil* elastase inhibitors[3] and inhaled nitric oxide[4]; and various methods of perfusion, such as antegrade cerebral perfusion[5]; pH and alpha stat management[6,7]; and pulsatile or low flow cardiopulmonary bypass[8]. The majority of studies have focused on one particular organ system: there are many studies examining neurological injury post cardiopulmonary bypass[9,10], several others looking specifically at renal injury[8,11] pulmonary injury[3,12], cardiac injury[13], and so on. However, the systemic inflammatory response to cardiac surgery is global and can affect all systems leading to multiple organ dysfunction. Thus using a multiple organ model of injury allowed me to assess the

mechanisms of the post cardiac surgical systemic inflammatory response and multiple organ dysfunction, and then using this knowledge, to assess the benefits of attenuating the inflammatory response through pre-conditioning with omega 3 fatty acids.

I conducted two studies – one group of ten animals (five control and five omega-3) were survived to eight hours; a second group of ten animals (again five control and five omega-3) were survived to 24 hours. In the eight hour study, one pre-operative dose of omega-3/control infusion was administered; while in the 24 hour study, two doses were administered, one for the four hours preceeding surgery, and one 24 hours prior to this. In the eight hour study, I focused predominantly on the clinical outcomes. The 24 hour study was then used to further examine the clinical outcome, but also to establish insight into the mechanisms of organ injury and the mechanisms of the protection conferred by omega-3 fatty acids.

2.1.2 Ethical Approval and Animal Care

All animal experiments were performed under a license obtained from the Department of Health under the Cruelty to Animals Act of 1876 (Appendix 1). Animal care conformed to institutional guidelines in compliance with international laws and policies. The protocols for these studies were approved by the Research Ethics Committee of the Royal College of Surgeons in Ireland (Appendix 1). All experiments were conducted in the Biomedical Research Facility, Beaumont Hospital, Dublin 9.

2.1.3 Juvenile Piglet Model of Cardiopulmonary Bypass and Circulatory Arrest

This model was established in the Biomedical Laboratory using a protocol designed by Dr John Byrne and Dr Jonathan McGuinness based on previous descriptions of the model in the literature and on the protocols and procedures used in Our Lady's Children's Hospital, Crumlin for the post operative care of paediatric cardiac surgical patients. Twenty five animals were used to establish all aspects of the model prior to commencing the study.

Surgical Preparation:

Male piglets weighing 10 – 15kgs at 4 weeks of age, ie fully weaned, were acclimated in a pig specific room in the Biomedical Facility for 2-4 days without any stresses. They had access to a standard pellet diet and water ad libitum. Due to their rapid intestinal transit times, a period of fasting for solid food of six hours only is necessary, with access to water provided until the time of surgery[14].

An intramuscular injection of midazolam (2mgs/kg) and ketamine (10mgs/kg) was given for sedation. The piglets were then weighed and transferred to the operating theatre. A pulse-oximeter sensor was attached to the ear for continuous monitoring of heart rate and oxygen saturations. Oxygen, at 1.5 – 2L flow as necessary to maintain oxygen saturations greater than 97%, and 2% isoflurane was delivered via a snout mask for maintenance of

anaesthesia. Depth of anaesthesia was monitored by eyelid reflexes and heart rate, and the animal re-dosed with two thirds the initial sedation dose of midazolam and ketamine after approximately two hours. An intramuscular injection of atropine (0.6ml) was given to reduce tracheal secretions. A rectal probe was inserted and temperature maintained at 38°C (normal porcine body temperature) using a heat lamp as necessary. Two peripheral ear vein cannulae were inserted for administration of maintenance intravenous fluids (Hartmann's at 4mls/kg/hr) and the control normal saline or omega-3 infusion (2ml/kg over 4 hours).

Following the four hour period of infusion, the piglet was prepared for cardiopulmonary bypass. ECG electrodes were attached. The sensors for the NIRS (Near InfraRed Spectroscopy) monitor were attached to shaved, cleaned and dried skin over the skull and the left renal angle for continuous monitoring of regional brain and renal cortical oxygen saturations. A diathermy pad was also applied. Under sterile conditions, a right femoral cut down was performed to access the femoral artery and vein. A triple lumen central line was placed for continuous monitoring of central venous pressure and for administration of anaesthetic agents, inotropes and fluid boluses as required during the experiment. A femoral arterial line was placed to allow continuous blood pressure and heart rate monitoring, and for blood sample and blood gas acquisition. Again under sterile conditions, a suprapubic urinary catheter was inserted directly into the bladder using a cut down technique to allow for accurate recording of hourly urine output and for sample collection. A bolus of intravenous fentanyl (250μg/kg) was then given and a tracheostomy performed for airway management. The isoflurane was then discontinued

and the piglet maintained under anaesthesia using intravenous fentanyl (25μg/kg/hr) and midazolam (0.2mg/kg/hr); pancuronium (0.2ml/kg/hr) was also administered for maintenance of paralysis to facilitate ventilation and surgery. The piglet was ventilated with a pressure controlled ventilator and a continuous FiO_2 of 1.0. The endotracheal tube was connected to the CO_2SMO Plus Respiratory Profile System (Novametrix, Wallingford, CT) for lung function measurements. Using the readings thus obtained for tidal volumes, the ventilator minute volume was adjusted to achieve tidal volumes of 150mls per breath for piglets up to 15kgs, and volumes of 10mls/kg for piglets greater than 15kgs (this was to account for dead space in the ventilator circuit), with a respiratory rate of 14 breaths per minute and a PEEP of 2. A nasopharyngeal temperature probe was also positioned at this time. Once this setup was completed, the piglet was left undisturbed for 10 minutes and a baseline set of readings, blood samples and blood gases were recorded. Arterial blood pressure during this period was maintained at greater than a mean of 60mmHg, with fluid boluses of 10mls/kg of Hartmanns if necessary, to keep CVP greater than 7mmHg.

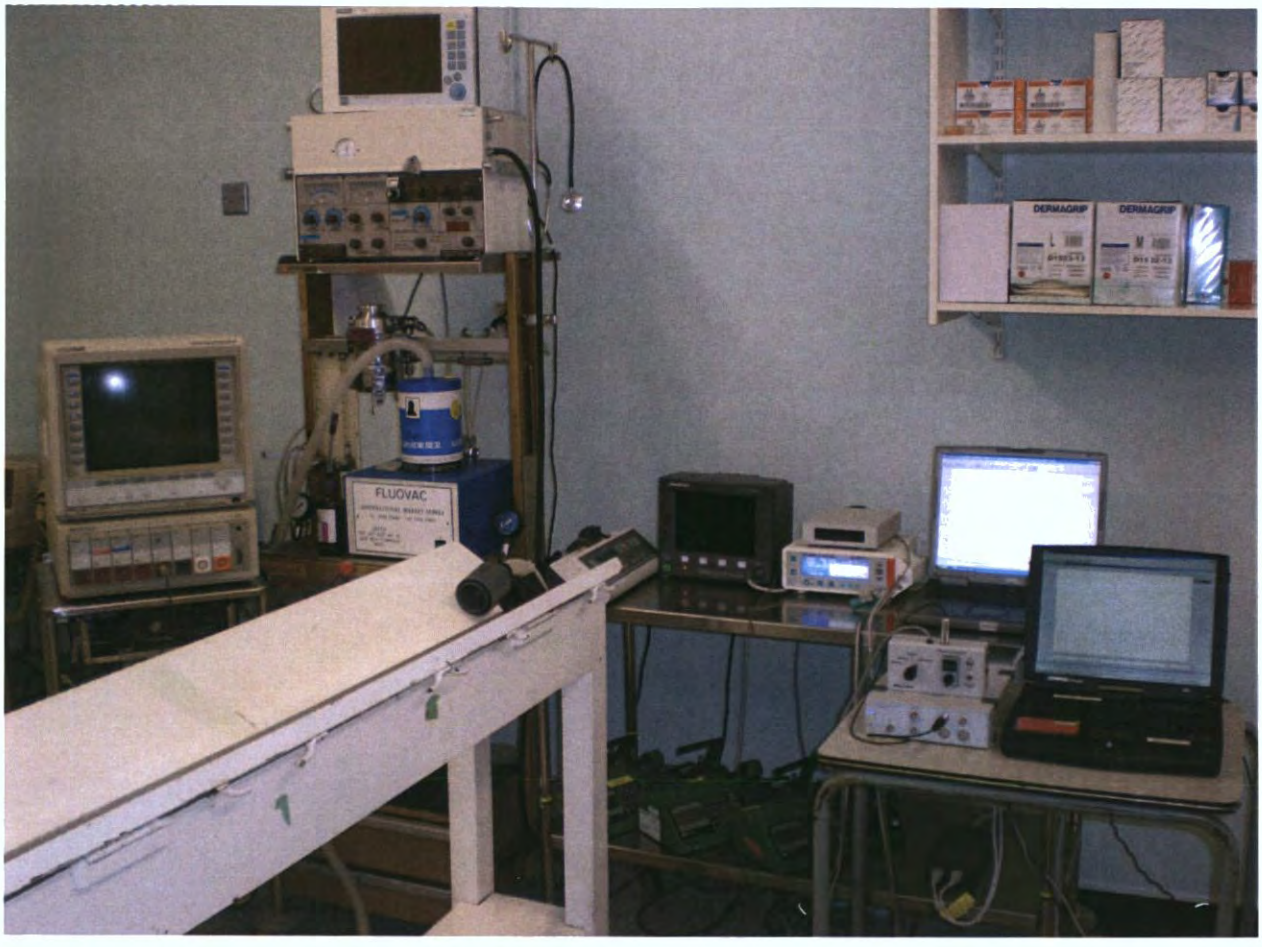

Image 1: Laboratory set-up: On the left, the monitor for temperature, MAP, CVP, and ECG trace. The ventilator is next to this. The NIRS monitor and the COSMO monitor and computer can be seen to the right of this on the table nearest the wall, with the cardiac catheter equipment seen in front of this.

Image 2: Cardiopulmonary bypass pump.

Cardiopulmonary Bypass:

A complete aseptic technique was used at all times during the cardiopulmonary bypass run. Following bethadine skin preparation and draping, a median sternotomy was performed, the thymus removed and the pericardium opened. Heparin (3mgs/kg) was given intravenously, with further doses if required, to obtain an ACT (activated clotting time) of >300 seconds prior to cannulation, and >480 prior to initiation of cardiopulmonary bypass. The aorta was then cannulated with a 12 French Jostra arterial cannula (Maquet), and the right atrial appendage with a 24 French standard metal angled tip DLP cannula (Medtronic). A baseline reading of cardiac contractility was then obtained using the Millar cardiac catheter inserted directly into the left ventricular apex. The cardiopulmonary bypass circuit used was the standard circuit used in the cardiac surgical department of Our Lady's Children's Hospital, Crumlin (Medtronic Minimax). We used a roller pump and a heat exchanger for temperature regulation. The prime consisted of 480mls of porcine whole blood, with 2000 IU of heparin and 1ml/kg of 8.4% sodium bicarbonate added. Porcine blood antigens are extremely varied, but weakly expressed, and transfusion reactions in previously un-transfused pigs do not seem to occur[15]. The porcine blood was obtained from a donor adult pig from the same herd the day prior to the experiment. The adult pig was anaesthetized with intramuscular ketamine (20mgs/kg) and midazolam (4mgs/kg). An endotracheal tube was placed to assist spontaneous breathing and to facilitate delivery of 2% isoflurane gas to maintain anaesthesia. Using a sterile technique, the carotid artery was identified and cannulated,

and the blood collected in citrate phosphate dextrose solution (Baxter Healthcare). The blood units were then stored at 4°C and used within a maximum period of 2 weeks.

Once cannulated and connected to the circuit, cardiopulmonary bypass was instituted with a flow rate of 100mls/kg, and the ventilator then disconnected. The oxygenator gas was pure oxygen, and the gas flow was adjusted to achieve a PCO_2 of 4-5.5kPa, with blood gases checked every fifteen minutes. An alpha stat technique was used. Venous drainage was by gravity. Hematocrit on bypass was maintained at 20 – 25%, and the ACT maintained at >480 seconds with additional heparin boluses if necessary. The piglet was maintained on normothermic bypass for one minute, and then cooled to a nasopharyngeal temperature of 18°C, maintaining a gradient (venous to heat exchanger and arterial to nasopharyngeal) of no more than 10°C while cooling and re-warming. Once the temperature was below 35°C, the aorta was cross clamped, and 20mls/kg of cold crystalloid cardioplegia (500mls of Ringer's Lactate, 12mls of 8.4% sodium bicarbonate, 10mls of cardioplegia concentrate - Martindale) was delivered antegradely through the aortic root via a 20g needle, with a second half dose if necessary to completely arrest the heart. Cold slush (frozen Ringer's lactate/0.9% saline) was applied topically to the heart, and ice packs were placed around the head and peripheries of the piglet. Once the 18°C target temperature was reached, the circulating volume was drained into the venous reservoir, and the bypass circuit switched off. The intravenous anaesthetic agents were also discontinued at this time. A ninety minute period of deep hypothermic circulatory arrest was then observed. A repeat dose of cold crystalloid cardioplegia was given as above after 45 minutes, and the cold slush replaced.

After ninety minutes, cardiopulmonary bypass was recommenced. The icepacks were removed, the anaesthetic agents restarted and the urinary bladder emptied (this was to allow for accurate hourly recordings from this time point on). The piglet was rewarmed, again maintaining the 10°C gradient as described for cooling. The cross clamp was removed after 1 minute of reperfusion on bypass. Just prior to removal of the aortic cross clamp, lignocaine (1mg/kg) and magnesium (400µl) were added to the circuit. Once the piglet reached >32°C, the heart was defibrillated if not in sinus rhythm at this point. Prior to discontinuing bypass, the lungs were re-inflated manually and then reconnected to the ventilator. Also at this time, dopamine (5mcgs/kg/hr) was commenced intravenously. When the piglet reached a nasopharyngeal temperature of 37°C or a core temperature of 35°C, and was hemodynamically stable and in sinus rhythm, bypass was weaned and then discontinued. The heart was de-cannulated and intravenous protamine (1mg/100iu of heparin) given, with additional doses as necessary to return the ACT to <140seconds. Hemostasis was secured, two 14g cannulae inserted through the chest wall into the pleural spaces as chest drains, the Millar cardiac catheter was inserted into the left ventricular apex, and the chest closed.

Post-operative Management:

8 hour model:

Post-operatively, the piglet was maintained on the same anaesthetic regime and mechanically ventilated for eight hours as described for the pre-operative period. The

mean arterial blood pressure was maintained at >60mmHg. This was achieved initially through boluses (10mls/kg) of pump blood during the first four hours, and Hartmann's during the second four hours to achieve a CVP of >6mmHg. If the MABP did not improve despite this, dopamine was re-commenced at 5mcg/kg/hr and titrated up as necessary. During the period of observation, hemodynamic and lung function measurements were recorded hourly, arterial blood gases two hourly, and blood and urine samples taken for future analysis as will be detailed below. At eight hours, the piglet was euthanized with intravenous sodium pentobarbital (100mgs/kg).

24 hour model:

Post-operatively, the piglet was maintained exactly as for the 8 hour model as described above for the first eight hours, in order to allow for accurate comparison between our 8 hour and 24 hours groups. At eight hours, the anaesthetic regime was changed from intravenous fentanyl and midazolam to intravenous propofol (2mgs/kg/hr). Pancuronium was continued at the same dose (2mls/kg/hr) for the full 24 hour observation period. Hemodynamically, the mean arterial pressure was maintained at 55 – 60mmHg and CVP at 10 – 12mmHg. This was achieved with fluid boluses initially. However, if CVP was 10 – 12mmHg and blood pressure remained low, noradrenaline (3mgs in 50mls of 0.9% saline) was commenced at 3mls/hr and titrated up to a mean arterial blood pressure of 50 – 60mmHg.

If acidosis developed, as measured by base excess of less than -6, bicarbonate was administered as a stat dose of 1mmol/kg.

From a ventilatory point of view, mean tidal volumes were maintained at 150mls for piglets up to 15kgs, and 10mls/kg for piglets greater than 15kgs. If oxygen saturations were less than 94% or pO_2 was less than 10 on arterial blood gas, the following steps were taken: firstly, we ensured that the piglet was not fighting the ventilator – pancuronium boluses of 1ml were administered as necessary to ensure adequate paralysis; secondly, we ensured that the tidal volume was correct; thirdly, if $pCO_2 > 6$, respiratory rate was increased to 18 and minute volume was simultaneously increased to maintain the appropriate tidal volumes; fourthly, if pO_2 was still low, we increased the PEEP to 8. The drains were also frequently aspirated to ensure there was no pneumothorax or hemothorax.

Again, during the entire period of observation, hourly observations were recorded. Blood and urine samples were collected and stored at various time points as will be detailed in the individual results sections.

At 24 hours, the piglet was euthanized with an overdose of sodium pentobarbital (100mgs/kg).

Organ Harvest:

Once death was confirmed, the chest was re-opened, the ascending and descending aorta clamped, and the superior vena cava transected. A 20g needle was then inserted into the ascending aorta and the brain infused with 1L of chilled 0.9% saline and then 1L of 4% paraformaldehyde in 0.1 molar phosphate buffered saline (PBS), pH 7.4 (Sigma) to fix

the brain in situ. While this was infusing, samples of the left lower lobe of the lung, the left ventricle, the left lower pole of the kidney, the small bowel and the left lobe of the liver were harvested. One sample of each was fixed in formalin and then embedded in paraffin 48 hours later for histology; one sample was snap frozen in liquid nitrogen for measurement of NFkB levels; and one sample was weighed, then dried in a convection oven at 80°C for 72 hours for calculation of wet:dry ratio as a measure of organ oedema. The brain and cerebellum were then harvested in total and stored in 4% paraformaldehyde for 24 hours, after which time the storage solution was changed to 30% sucrose in 0.1 molar PBS, PH 7.4 (Sigma).

2.1.4 Pre-treatment Protocol:

8 hour model:

Alternate piglets were assigned to the control or the omega-3 infusion group. As described above, the animals were sedated and monitored, and the infusion administered through a peripheral ear vein cannula for the four hours immediately prior to pre-operative preparation for cardiopulmonary bypass.

The control group received 2mls/kg of 0.9% saline (Baxter, UK) over a four hour period.

The omega-3 group received 2mls/kg of Omegaven over a four hour period. Omegaven (donated by Fresenius Kabi, Germany) is a fatty acid infusion licensed for clinical use via

a peripheral vein as part of total parenteral nutrition regimes. It contains predominantly the long chain fatty acids eicosapentaneoic acid and docosahexaenoic acid, but also smaller quantities of omega-6 fatty acids and saturated and monounsaturated fatty acids, as shown in table 1 below. The infusion also contains a small amount of Vitamin E (alpha tocopherol) as an antioxidant. It has a pH of 7.5 – 8.7, and an osmolality of 308 – 376 milli-osmoles/ml (Fresenius Kabi, Sept 1998). The recommended daily dose for clinical use of 2mls/kg body weight was used, and it was given over the recommended time period of four hours.

Component	*Concentration per 100mls*
Eicosapentaenoic Acid (EPA)	1.25-2.82g
Docosahexaenoic Acid (DHA)	1.44-3.09g
Myristic Acid	0.1-0.6g
Palmitic Acid	0.25-1.0g
Palmitoleic Acid	0.3-0.9g
Stearic Acid	0.05-0.2g
Oleic Acid	0.6-1.3g
Linoleic Acid	0.1-0.7g
Linolenic Acid	<=0.2g
Octadecatetraenoic Acid	0.05-0.4g
Eicosaenoic Acid	0.05-0.3g

Arachidonic Acid	0.1-0.4g
Docosaenoic Acid	<=0.15g
Docosapentaenoic Acid	0.15-0.45g
dl-alpha-Tocopherol	0.015-0.0296g
Glycerol	2.5g
Purified egg phosphatide	1.2g

Table 1. Composition of Omegaven (Fresenius Kabi)

24 hour model:

In the 24 hour study, piglets received two four hour pre-operative infusions: one for the four hours just prior to the pre-operative preparation for cardiopulmonary bypass (exactly as for the 8 hour model), and a second infusion 24 hours prior to this. The second dose of omega-3 was added in the 24 hour model following analysis of the 8 hour study, where some beneficial effects were noted (see results section) – it was hoped that a second dose would maximise these benefits. In addition, the previous work carried out in our laboratory using the rabbit regional ischemia model which showed a 40% reduction in myocardial infarct size had a four day pre-operative omega-3 infusion regime. For the earlier infusion, the piglets were sedated and monitored in the same way as previously described. In brief, they received an intramuscular injection of midazolam (2mgs/kg) and ketamine (10mgs/kg). A snout mask delivered 2% isflourane to maintain anaesthesia, and 1.5 – 2L of oxygen to maintain oxygen saturations at greater than 97%. An intramuscular injection of atropine (0.6mls) was administered to reduce tracheal secretions and

temperature was maintained at 38°C. A peripheral ear vein cannula was inserted and the control or omega-3 infusion administered over a four hour period. After two hours, the piglets were re-dosed with two-thirds the original dose of ketamine and midazolam. Following the four hour infusion, the cannula was removed and hemostasis ensured. The isoflurane was then discontinued, and the piglets were observed until they had woken and returned to eating and drinking.

The control group received two infusions of 2mgs/kg of 0.9% saline over four hours, at the time points described above. The omega-3 group received two infusions of 2mgs/kg of Omegaven over four hours at the same time points.

Primary Endpoints – Clinical Outcomes

2.1.5 Assessment of Hemodynamic Stability:

A number of parameters, as described in the PRIMACORP study (prophylactic intravenous use of milrinone after cardiac operation in pediatrics)[16], were recorded hourly in order to assess post operative hemodynamic status and to observe for the development of a low cardiac output state. Heart rate and blood pressure were recorded, as measured by the femoral arterial line; central venous pressure, as measured by the femoral central venous line; hourly urine output, taken from the suprapubic catheter; and regional renal cortical and cerebral oxygen saturations, as measured by the NIRS probes (see below).

The number of fluid or blood boluses and the amount of dopamine required post-operatively to maintain a mean arterial blood pressure of greater than 60mmHg with a CVP of greater than 6mmHg was recorded. In addition, two hourly arterial blood gases were analyzed and lactate, base excess, and mixed venous oxygen saturations were documented, as indicators of tissue perfusion.

NIRS (Near Infra Red Spectroscopy) (INVOS, Somanetics, Troy, MI) is a non-invasive method of quantifying regional tissue perfusion. The skin sensors send near infrared light into the tissues at two depths in an arc which measures the mean oxygenation state of the haemoglobin passing through that area. Using two depths of penetration allows a reading to be obtained for the superficial skin, fat and muscle or bone, and one which includes these structures and also the kidney/brain at a deeper level. The two values are then automatically subtracted to give a reading for the kidney/brain. The value thus obtained represents a mix of arterial and venous blood in an approximate 3:1 ratio.

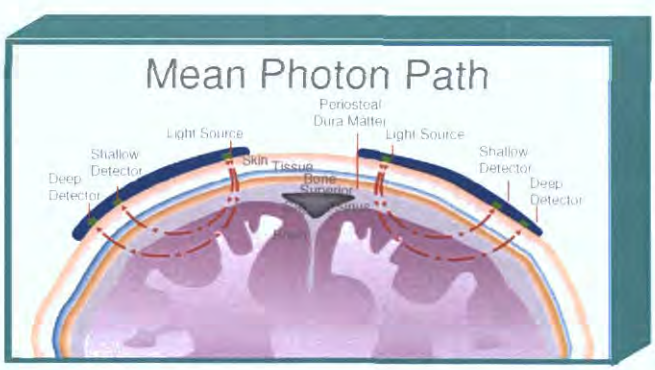

Figure 1: Mechanism of measurement of regional cerebral oxygen saturations using NIRS

The normal baseline adult cerebral regional oxygen saturation is approximately 70%. In children with congenital cardiac disease without left-to-right shunting, the baseline value has been shown to be similar to adults; however, in those with left-to-right shunting, it is significantly lower[17]. In general, trends are monitored more than absolute values, and a reduction of more than 20% from baseline is considered significant[18].

As this is a simple, non-invasive mechanism of cerebral and somatic regional oxygen saturation, much interest and study has been generated as to its place in the management of cardiac patients, particularly in paediatrics. The readings obtained have been shown to correlate well with mixed venous oxygen saturations obtained through invasive monitoring[19]; and with jugular bulb venous saturations[20]. It has also been used in studies to determine critical periods of reduced oxygenation during cardiopulmonary bypass and circulatory arrest[21,22]. The clinical literature suggests a correlation between low regional oxygen saturations and adverse neurological outcomes[17,23], but overall there is a lack of strong evidence to support clinical decision making based on NIRS data alone[24,25]. In this study, it was used as an additional marker of a low cardiac output state.

2.1.6 Assessment of Cardiac Injury:

Millar cardiac catheter assessment of left ventricular function:

A 2.2 French Millar cardiac catheter (SPR-249A, Millar Instruments Inc, Texas, USA) was used to measure left ventricular function. At the time of operation, just prior to commencing bypass, the left ventricular apex was punctured using a 20guage needle and the catheter inserted for baseline readings of left ventricular end systolic and end diastolic pressures, and also the maximum and minimum rate of change of pressures (dP/dt_{max} and dP/dt_{min} respectively). The catheter was then removed for the period of DHCA. At chest closure, the catheter was threaded through the skin using a 16guage cannula and advanced into the left ventricle, where it remained for the 8 hour period of observation. Recordings were made at baseline, and then at two hourly intervals following recommencement of cardiopulmonary bypass. At each recording, it was ensured that mean arterial pressure was greater than 60mmHg and CVP was greater than 6. Also a standardised per weight anaesthetic regime was used and temperature was maintained at 38°C. This was to minimise preload and afterload variability, as dP/dt_{max} and dP/dt_{min} are preload and afterload dependant, and therefore will follow Starling Curve dynamics. Each recording was for thirty seconds, and readings were taken at three time points during the recording, with averaging of three consecutive pressure complexes for each reading. The readings were processed using Powerlab 2/20 (AD Instruments, Oxfordshire, UK) and Chart v4.04 on a Microsoft ME operating system.

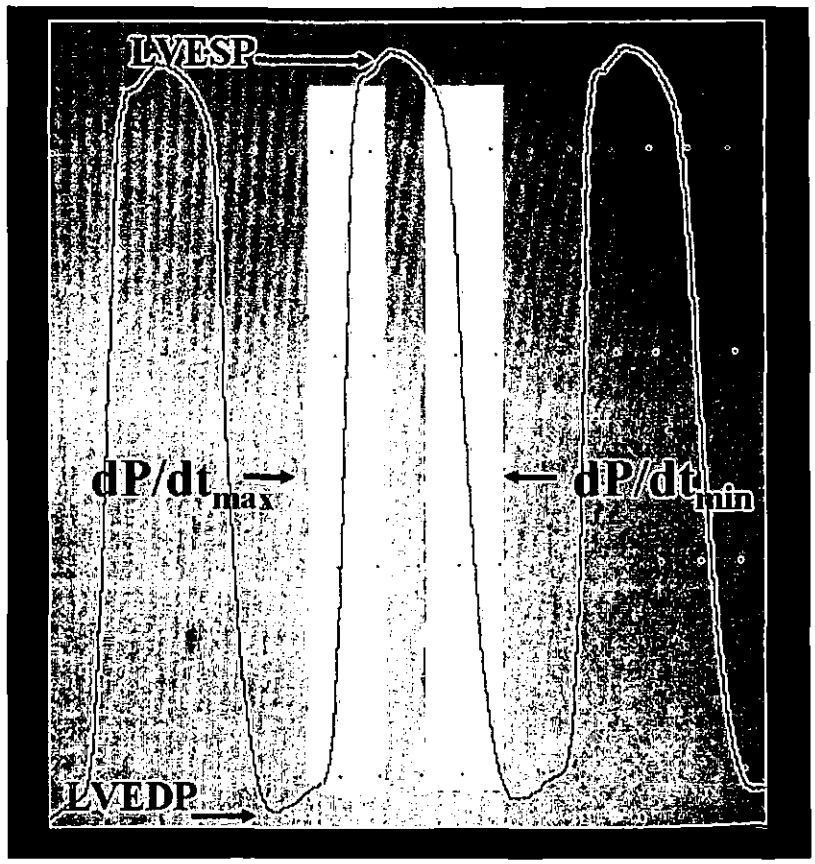

Figure 2: This trace shows the four readings of left ventricular function taken with the cardiac catheter: LVEDP (left ventricular end diastolic pressure); LVESP (left ventricular end systolic pressure); dP/dt_{max} (the rate of change of pressure within the ventricle during systole); and dP/dt_{min} (the rate of change of pressure within the ventricle during diastole).

Serum Troponin I:

Troponin I is a well established serum marker of myocardial injury. Serum troponin was measured on an analyzer by the biochemistry laboratory in Beaumont Hospital (UniCel DXI 800, Beckman Coulter, California, USA) on samples taken at baseline and at three hours post recommencement of bypass, time points used to detect the greatest change in troponin as described previously with the juvenile piglet bypass model[26]. It was also measured at 6, 12, 18 and 24 hours in our 24 hour model.

2.1.7 Assessment of Pulmonary Injury:

Respiratory function was assessed using arterial blood gases, and the CO_2SMO Plus respiratory profile system (Novametrix, Wallingford, CT) as has been previously described with the juvenile piglet model[2].

CO_2SMO Plus:

The CO_2SMO Plus monitor is attached to the endotracheal tube and provides continuous in-line monitoring of a number of respiratory parameters. Static and dynamic compliance of the lungs and dynamic inspiratory and expiratory airway resistances were recorded at baseline and on an hourly basis following recommencement of bypass.

PO_2:FiO_2 Ratio:

Using two hourly arterial blood gases, the ratio of arterial oxygen tension (PaO_2) to fractional inspired oxygen (FiO_2) was calculated. The PaO_2:FiO_2 ratio is a widely used oxygenation index; Acute Respiratory Distress Syndrome (ARDS) is defined as a PO_2:FiO_2 ratio of less than 200[27].

A-a Gradient:

The alveolar: arterial gradient (A-a gradient), a measure of alveolar capillary gas exchange, was also calculated using the formula: A-a gradient = $P_AO_2 - PaO_2$; where $P_AO_2 = (FiO_2 \times (760 - 47)) - (PaCO_2/0.8)$; where 760mmHg is atmospheric pressure, 47mmHg is the partial pressure of water vapour (as alveolar gas is completely saturated with water), and 0.8 is a constant, the respiratory quotient.

2.1.8 Assessment of Renal Injury:

Renal function was assessed looking at markers of injury related to specific portions of the nephron in order to define the location and mechanism of injury post cardiopulmonary bypass.

Creatinine Clearance:

Creatinine clearance (CrCl) is the volume of blood plasma that is cleared of creatinine per unit of time, and is a surrogate marker of the glomerular filtration rate (GFR). Creatinine

is freely filtered at the glomerulus, but it is also actively secreted by the tubules in very small amounts; thus the creatinine clearance overestimates the actual GFR by approximately 10 -20%. This is considered acceptable due to the ease by which CrCl can be calculated, rather than the use of inulin clearance (a more accurate method of estimation of GFR). The normal value of CrCl is variable, but should be approximately 75 – 125mL/min[28]. Creatinine clearance is usually measured over 24 hours, however in this study, it was calculated two hourly indexing to serum creatinine in order to correct for hourly variations in urine output. The following formula was used: CrCl = (urinary creatinine x average hourly urine output per kilogram over the previous two hours x 1.16) / serum creatinine.

Urinary lysosomal N-acetyl-beta-glucosaminadase (NAG) levels:

Urinary NAG is a specific proximal tubular lysosomal enzyme. It is not filtered at the glomerulus, nor is it absorbed or secreted by the tubules; therefore, increases in the urinary concentration of NAG are indicative of tubular cell damage[28]. A study by Lockwood et al[29] demonstrated that in urine, 0.2% of the activity was lost per hour at 37°C and that the activity was not denatured by physiological variations in urinary pH or osmolarity. In addition, the rate of excretion is independent of body mass. In this study, samples were all aliquoted and frozen at -80°C within 45 minutes of collection. There are no specific porcine kits for the measurement of NAG, nor has it been previously assessed in the juvenile piglet model. However, we used a calorimetric assay designed for human urine samples to measure this on a two hourly basis in our model (Cat No 10875406001, Roche Diagnostics Ltd, UK) - as this is a calorimetric assay, the manufacturers expected

that it would be compatible with the measurement of NAG in porcine samples. The results were calculated as ratio to urinary creatinine concentration at each time point in order to correct for variations in urinary flow rates, as described in the literature [27,30].

Fractional Excretion of Urinary Sodium:

Fractional excretion of urinary sodium is a measure of the percentage of sodium excreted in the urine compared to the sodium reabsorbed by the kidney, and is used as a measure of distal renal tubular function. The measurement of FeNa has been shown to be a significant diagnostic factor for acute renal failure[31]. A level of greater than 1% indicates the loss of the reabsorption capacity of the tubules[32], thus suggesting acute tubular necrosis or other intrinsic cause of renal impairment; while a low value indicates sodium retention by the kidney, which may be the physiological response to a reduction in renal perfusion such as occurs with hypovolemia, and can be indicative of the pre-renal nature of an ARF. In this study, it was measured at baseline and two hourly following recommencement of bypass using the following standard formula: {(Urinary sodium/Serum sodium) x (Serum creatinine/Urinary creatinine)} x 100. The result obtained can be affected by diuretic use, as many of these drugs interfere with the way the kidney handles sodium; diuretics were not used during this study.

Urine Output:

As an overall measure of renal function, we measured hourly urine output per kilogram body weight.

2.1.9 Assessment of Cerebral Injury

Cerebral injury is the most widely studied endpoint of research using the juvenile piglet cardiopulmonary bypass model. The neocortex and the CA1-4 regions of the hippocampus are the major areas injured following bypass and circulatory arrest, and this injury is mediated both by necrosis and apoptosis[33,34]. Recovery juvenile piglet bypass models have correlated clinical neurological status with histological injury[35,36].

Regional oxygen saturation in the cerebral cortex with NIRS monitoring:

In our model, continuous monitoring of cerebral cortical regional oxygen saturations was employed, with recordings taken at baseline and hourly following recommencement of bypass.

Histology:

As described above, the brain and cerebellum were harvested en bloc following in situ fixation with chilled 0.9% saline and 4% paraformaldehyde in 0.1 molar phosphate buffered saline (Sigma Chemicals). They were then stored in a 4°C fridge in 4% paraformaldehyde in phosphate buffered saline for 24 hours, after which time the storage solution was changed to 30% sucrose (Sigma) in phosphate buffered saline.

The cerebral injury was assessed by Dr Manus Ward in the Neurophysiology Department of the Royal College of Surgeons in Ireland. Specifically, the CA 1-4 regions of the hippocampus were examined histologically. Sections 4 μm in thickness were cut from blocks and floated onto glass slides. Sections were then allowed to dry. Once dried they were then automatically stained on a Varistain Gemini automatic stainer (Thermo Shandon, Cheshire, UK) using the pre-set H&E

staining protocol. Briefly, following dewax and rehydration, sections were stained in Mayer's haematoxylin for 2 minutes (Cellpath, Mid Wales, UK), differentiated in 0.125% acid alcohol, and stained in 1% aqueous Eosin (GCC Diagnostics, Flintshire UK) for 1.5 minutes. Sections were then dehydrated in alcohols, cleared in Ultraclear (J.T. Baker) and a glass coverslip was secured over the section using EZ Mount (Thermo Shandon, UK).

The sections were then assessed for neuronal necrosis and apoptosis, and signs of inflammation.

Secondary Endpoints – Mechanistic insight

2.1.10 White Cell Counts

Blood samples taken from the arterial line were analyzed by the Haematology Laboratory of Beaumont Hospital for white cell count and differential (Advia 2120 Hematology Analyzer, Global Medical Instrumentation, Minnesota, USA) at baseline, and then at 15 minutes, 30 minutes, 1 hour and 6 hours post recommencement of bypass.

2.1.11 ELISA Measurements

To quantify relevant cytokines of the systemic inflammatory response, commercially available kits which employ the sandwich enzyme immunoassay technique were used. In

brief, a monoclonal antibody specific for the factor being measured has been pre-coated onto a microplate. Standards, controls and samples are then pipetted into the wells and any of the factor present in the samples is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for the factor being measured is added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution is added to the wells. This produces a colour in proportion to the amount of the factor which is bound in the well. The intensity of the colour is measured using a microplate reader at a specific wavelength, and the sample values are then read from the constructed standard curve.

Serum and plasma samples were taken at baseline and at two hourly intervals from the femoral arterial line. Plasma samples were centrifuged immediately for 15 minutes at 1000g at 16°C. Serum samples were allowed to clot for fifteen minutes and then centrifuged for 10 minutes at 1500g at 4°C. The samples were then stored at minus 80°C in 300μL aliquots until assayed.

All assays were performed in duplicate.

Interleukin-6 (IL-6):

The Quantikine Porcine IL-6 Immunoassay (P6000, R&D Systems, UK) was used to measure levels of IL-6 in plasma samples. The minimum detectable dose of porcine IL-6 with this kit is 10pg/mL. Optical densities were measured at a wavelength of 450nm to

detect sample concentrations, with correction for optical imperfections in the plate obtained by subtracting readings obtained at a wavelength of 595nm. A standard curve plotting optical density against IL-6 concentration on a logarithmic scale was used to extrapolate unknown sample concentrations as per the manufacturer's instructions.

Interleukin-8 (IL-8):

The Quantikine Porcine IL-8 Immunoassay (P8000, R&D Systems, UK) was used to measure IL-8 in serum samples. The minimum detectable dose of IL-8 using this kit is reported as a mean value of 4.6pg/mL. Optical densities were measured as in the IL-6 assay, and a standard curve was constructed from which sample concentrations were read.

Interleukin-10 (IL-10):

The Quantikine Porcine IL-10 Immunoassay (P1000, R&D Systems, UK) was used to measure IL-10 levels in serum samples. The mean minimum detectable dose with this kit is 3.5pg/mL. As with the IL-6 assay, optical densities were measured at 450nm and 550nm, and a standard curve generated from which sample concentrations were read.

Leukotriene B_4 (LTB_4):

The Parameter LTB_4 Assay was used to measure LTB_4 levels in plasma samples (KGE006B, R&D Systems, UK). This is a multispecies kit which detects a mean minimum concentration of 8.2pg/mL. Optical densities were measured as for the IL-6

assay, and a standard curve generated on a logarithmic scale from which sample concentrations of LTB_4 were read.

2.1.12 Organ Histology

As described above, at the end of the observation period, samples of heart, lung, kidney, liver and gut were harvested, with one sample fixed in 10% formalin for 48 hours, followed by automatic dehydration in a graded series of alcohols overnight, and then paraffin embedded. Slices were cut and the slides stained for hematoxylin and eosin by the Pathology Department of Beaumont Hospital according to the following protocol. Sections 4 μm in thickness were cut from blocks and floated onto glass slides. Sections were then allowed to dry. Once dried they were then automatically stained on a Varistain Gemini automatic stainer (Thermo Shandon, Cheshire, UK) using the pre-set H&E staining protocol. Briefly, following dewax and rehydration, sections were stained in Mayer's haematoxylin for 2 minutes (Cellpath, Mid Wales, UK), differentiated in 0.125% acid alcohol, and stained in 1% aqueous Eosin (GCC Diagnostics, USA) for 1.5 minutes. Sections were then dehydrated in alcohols, cleared in Ultraclear (J.T. Baker) and a glass coverslip was secured over the section using EZ Mount (Thermo Shandon, UK). The organs were graded for neutrophil infiltration, and markers of tissue damage by two blinded pathologists. The scoring system used (detailed below) was designed specifically by the pathologists based on a number of papers in the literature[37, 38].

HISTOLOGICAL SCORING SYSTEM

Necrosis

0 = no changes

1 = up to 10% of cells affected

2 = 11-25% of cells affected

3 = >25% of cells affected

Cellular degeneration

(nuclear swelling, pyknosis, vacuolation)

0 = no changes

1 = up to 15% affected

2 =16-50% affected

3 = >50% affected

Inflammatory cell infiltration

0 = no changes

1 = up to 15% of area affected

2 = 16-50% of area affected

3 = >50% of area affected

Oedema

0 = no changes

1 = up to 10% of area affected

2 = 11-25% of area affected

3 = >25% of area affected

Hemorrhage

0 = no changes

1 = up to 10% of area affected

2 = 11-25% of area affected

3 = >25% of area affected

Vessels

Endothelial activation (swelling and proliferation)

0 = no changes

1 = changes in up to 3 arteries

2 = changes in >3 arteries

3 = changes in most arteries

Obliteration/thrombosis

0 = no changes

1 = 30% obliteration/thrombosis of any artery

2 = 50% obliteration of any artery or thrombosis in 2-3 arteries

3 = total obliteration of any artery or thrombosis in >3 arteries

Vasculitis

0 = no changes

1 = 1-3 leukocytes in any vascular wall

2 = 3-6 leukocytes in any vascular wall

3 = >6 leukocytes in any vascular wall

Cytoplasmic vacuolation

0 = no changes

1 = up to 15% of area affected

2 = 16 – 50% of area affected

3 = >50% of area affected

Interstital thickening (lung)

0 = no changes

1 = mild thickening of more than 25% of area

2 = moderate thickening of more than 25% of area

3 = severe thickening of more than 25% of area

% of area affected calculated by the average estimation over 5 high power fields (40x magnification)

2.1.13 Myeloperoxidase Measurement

Myeloperoxidase (MPO) is an enzyme released by activated neutrophils sequestered in the tissues, and therefore can be used as an indirect marker of the degree of neutrophil sequestration in the tissues. Immunohistochemical myeloperoxidase staining was performed by the Pathology Department, Beaumont Hospital, on slides cut from paraffin embedded samples of the heart, lungs and kidneys as described below. A human myeloperoxidase antibody (polyclonal rabbit anti-human myeloperoxidase, Cat No: A0398, Dako, UK) was used. This antibody has been shown by the company to cross react with the myeloperoxidase equivalent protein in swine.

Sections 4 μm in thickness were cut from the paraffin embedded blocks and floated onto adhesive slides (Leica Microsystems Plus Slides, Wetzlar, Germany). Sections were then baked at 37°C overnight. All staining was carried out on a BondMax automated

immunostainer from Leica Microsystems. Sections were loaded onto the system and the relevant program was started. The BondMax sytem dewaxed slides and then carried out the appropriate antigen retrieval technique that was previously optimised for MPO (ER1 20 minutes). The myeloperoxidase primary antibody (polyclonal rabbit anti-human myeloperoxidase, Cat No: A0398, Dako, UK) diluted 1 in 200 in Bond Primary Antibody Diluent (Leica Microsystems, Germany) was automatically added to the sections for 20 minutes. Following detection DAB (diaminobenzidine) was used as the chromagen and sections were then counterstained lightly with haematoxylin, and processed to coverslip.

The stained sections were then assessed by our blinded pathologists. MPO staining was counted over four high power fields.

2.1.14 Measurement of NFkB:

In order to measure NFkB using the commercially available ELISA-based kits, the nuclear and cytoplasmic proteins were first extracted from the organ samples using the TransAM Nuclear Extract Kit (Cat No: 40010, Active Motif Europe, Belgium) as per the manufacturer's instructions as follows.

Isolation of nuclear and cytoplasmic proteins:

Organ and muscle samples were harvested as detailed earlier and frozen at -80°C. The samples were homogenized on ice in complete lysis buffer (prepared fresh on the day of the experiment from the kit) and then incubated on ice for 30 minutes. They were then

centrifuged at 4°C at 4100g for 10 minutes. The supernatants were then transferred to pre-chilled microcentrifuge tubes and centrifuged at 10 000g for 10 minutes at 4°C. The supernatant was transferred to a new pre-chilled microcentrifuge tube and the process repeated. The samples were kept on ice at all times. The supernatant thus obtained was the whole-cell lysate.

The Bradford assay was used to determine the protein concentration in each sample. The Bradford reagent (Sigma Chemicals) was warmed to room temperature. A standard curve was generated from a bovine serum albumin standard (2mg/ml) diluted in the complete lysis buffer prepared from the nuclear extract kit. 5µL of protein standard or sample and 250µL of Bradford reagent were added to each well of a 96-well plate in duplicate. The samples were incubated for 20 minutes at room temperature and the absorbance then measured at a wavelength of 570nm using a microplate reader. The standard curve was plotted and the equation generated used to determine the protein concentrations in the samples. The sample concentrations were then diluted to 2µg of whole cell lysate per 20µL of complete lysis buffer for the subsequent ELISA.

Measurement of NFkB:

The samples prepared as above were then assayed for levels of NFkB (TransAm NFkB Family Kit, Cat No: 43296, Active Motif Europe, Belgium) as per the manufacturer's instructions. NFkB is a transcription factor important in the regulation of many genes that code for mediators of the inflammatory response. There are five subunits of the NFkB family in mammals: p50, p65 (RelA), c-Rel, p52, and RelB. The p50 and p65 subunits are the active subunits; therefore in this study, the p65 subunit was measured. The kit

used contains a 96-well plate to which oligonucleotide containing an NFkB binding sequence is immobilized. The activated NFkB in the nuclear extracts prepared as above specifically binds to this oligonucleotide. By then adding an antibody to the p65 subunit the NFkB complex bound to the oligonucleotide is detected. Addition of a horseradish peroxide conjugated secondary antibody provides a sensitive colorimetric readout that is quantified by spectrophotometry at a wavelength of 450nm. The reading obtained is then directly proportional to the amount of NFkB in the measured sample.

2.2 Statistical Analysis:

The statistical analysis used with each endpoint is described in each chapter with the results. I am very grateful for the statistical advice provided by Dr Kathleen Bennett (biostatistician) of Trinity College Dublin.

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CHAPTER 3

MECHANISMS AND PATTERNS OF ORGAN INJURY IN A 24 HOUR MODEL OF PAEDIATRIC CARDIAC SURGERY

3.1 Introduction

As discussed, cardiac surgery can produce multiple organ dysfunction post-operatively, particularly in the vulnerable young and elderly. This can range from a mild sub-clinical picture to serious multiple organ failure. In order to improve peri-operative management of cardiac surgical patients and to develop new organ protection strategies, a complete understanding of the pattern, timing and underlying mechanisms of injury in each organ is of vital importance. While historically, the multi-organ dysfunction seen was attributed to peri-operative hypoperfusion, it is now well established that the ischemia-reperfusion injury - both myocardial in the case of cardiopulmonary bypass with cardioplegic arrest, and global, particularly in the setting of deep hypothermic circulatory arrest - and the systemic inflammatory response to surgery and cardiopulmonary bypass also have a vital role. However, while all of these mechanisms undoubtedly play a role, the predominant mechanism of injury in each organ has yet to be completely defined.

The aim of this research therefore was to establish the pattern, extent, timing and underlying mechanism of injury in each organ following paediatric cardiac surgery using the juvenile piglet bypass model of cardiopulmonary bypass and circulatory arrest, a model extensively described in the literature as suitable for the study of paediatric cardiac surgical physiology.

3.2 Materials and Methods

The juvenile piglet model of cardiopulmonary bypass and circulatory arrest was used as detailed in section 2.1.1. For this study, eleven animals were used. Five animals (Group 1) received one four hour infusion of normal saline (2mls/kg) through a peripheral ear vein cannula immediately pre-operatively and were survived to eight hours post operatively. Five animals (Group 2) received two four hour infusions of normal saline (2mls/kg each) through a peripheral ear vein cannula, one immediately pre-operatively and one 24 hours pre-operatively, and were survived to 24 hours post-operatively. One animal received two four hour infusions of normal saline (2mls/kg each) as with the animals in Group 2 and was euthanized following the infusions - this animal did not undergo cardiopulmonary bypass and served as a sham control. The differences in the administered infusions between the two groups correspond to two different omega-3 pretreatment protocols as detailed in Chapters 4 and 5.

The protocol used was based on the literature descriptions of the juvenile piglet model and also on the practices of the paediatric cardiac surgical department of Our Lady's Children's Hospital, Crumlin. Each animal received his pre-operative infusion and non-invasive and invasive monitoring were then established. This included continuous ECG monitoring, cerebral and renal NIRS, rectal and nasopharyngeal temperatures, femoral arterial and venous lines (for the monitoring of heart rate, blood pressure, central venous pressure; and to facilitate sample collection), suprapubic catheter (for hourly urine output measurements and to facilitate sample collection), and endotracheal tube (to assist ventilation, and to allow for continuous monitoring of a number of parameters using the

CO_2SMO Plus respiratory profile monitor). Cardiopulmonary bypass was then established, the piglets were cooled, and at 18°C the circulation was arrested for 90 minutes. Following this, the piglets were rewarmed, weaned from bypass and the chest closed. The period of observation, either 8 or 24 hours, was then observed with continuous monitoring and support as described in Chapter 2.

Cardiac function was assessed using haemodynamic variables (recorded hourly), serum troponin measurements (at baseline, 3, 6, 12, 18, and 24 hours); and cardiac catheter recordings of dP/dt maximum and dP/dt minimum as measures of left ventricular systolic and diastolic function (recorded at baseline and two-hourly following the re-institution of cardiopulmonary bypass).

Pulmonary function was assessed using the CO_2SMO Plus respiratory profile monitor attached to the endotracheal tube. Static and dynamic compliance were recorded hourly and airway resistances recorded two hourly. An arterial blood gas was analyzed two hourly (sample taken from the femoral arterial line) and provided information for the monitoring of trends in pO_2; pO_2:FiO_2 ratio; and alveolar-arterial gradient.

Renal function was assessed in a number of ways. Renal NIRS readings were recorded hourly; urine output was recorded hourly; and samples of blood and urine were obtained at baseline and two hourly following the re-institution of bypass for the measurement of creatinine clearance; fractional excretion of urinary sodium; and urinary N-acetyl glucosaminidase.

Cerebral injury was assessed with hourly recordings of NIRS readings, and with histological examination of the brain removed en bloc at the termination of the experiment.

With regard to mechanistics, blood samples were taken for measurement of white cell counts at baseline, 15 mins, 30 mins, 1, 6, 12, 18 and 24 hours following reperfusion. In addition, plasma and serum samples were taken at baseline and two hourly after reperfusion, centrifuged and the supernatants stored at minus 80°C for later analysis of IL-6, IL-8, and IL-10. Samples of each organ were harvested at the end of the period of observation in both groups for histological examination; the measurement of wet:dry ratios (representing the level of organ oedema); and myeloperoxidase levels by immunohistochemistry (myeloperoxidase is a tissue damaging enzyme released by activated neutrophils). In addition, levels of the p65 subunit of NFkB were measured in each organ.

3.3 Results

3.3.1 Cardiac Injury

Ventricular function was measured using a Millar cardiac catheter inserted directly into the left ventricle. An initial significant increase in systolic function was observed; readings had returned to baseline levels by 8 hours following reperfusion and were stable then throughout the remainder of the observation period. Of note, the animals were weaned from cardiopulmonary bypass following DHCA on a continuous infusion of

dopamine; this was discontinued by approximately 3 hours. There was no statistically significant change in diastolic function over the 24 hours; however, there appeared to be a trend towards reduced dP/dt min suggesting a trend towards diastolic dysfunction.

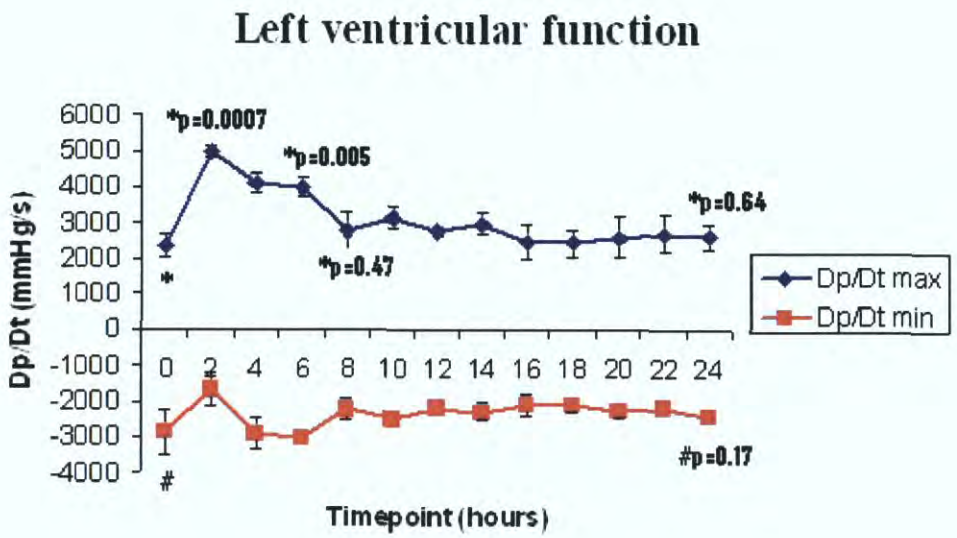

Figure 3.1

Left ventricular function was measured directly with a cardiac catheter at baseline and then two hourly following reperfusion. Results are reported as mean change in pressure with respect to time for systolic (dP/dt max, mmHg/s) and diastolic (dP/dt min, mmHg/s) function +/- SEM. Statistical analysis was with one way ANOVA and Bonferroni comparison of means. Systolic function showed a significant increase compared to baseline levels in the early post-operative period; this returned to baseline by 8 hours (p=0.47, Bonferroni comparison). Diastolic function was stable throughout the observation period (p=0.17, ANOVA), although a trend towards an initial increase can be seen on the graph.

Troponin T levels were measured by the Biochemistry laboratory in Beaumont Hospital on serum samples at baseline, 3, 6, 12, 18 and 24 hours. As can be seen in the graph, troponin levels peaked at 6 hours, and then steadily decreased over the following 18 hours. However levels were still significantly above baseline at 24 hours.

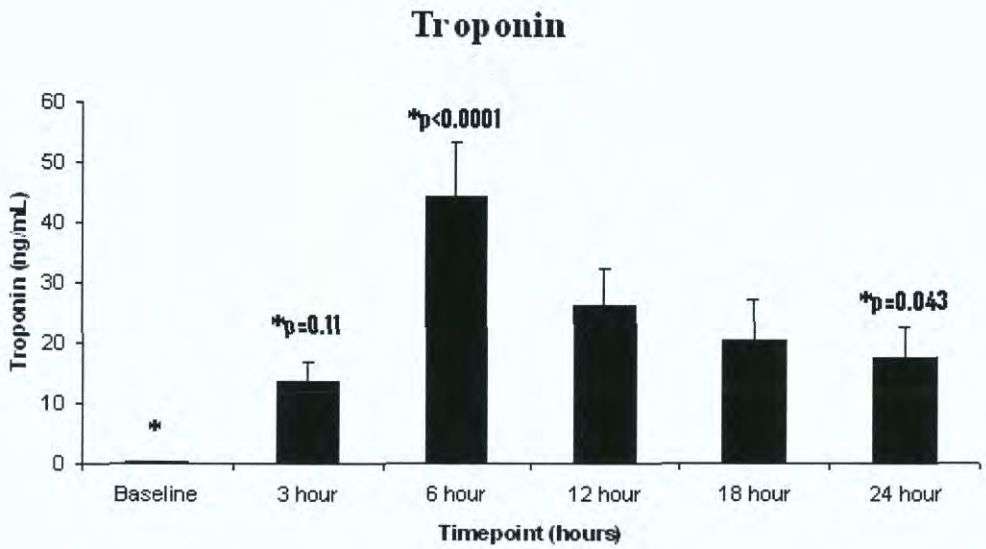

Figure 3.2

Troponin levels were measured at baseline and then at 3, 6, 12, 18, and 24 hours following reperfusion. Results are reported as mean troponin (ng/mL) +/- SEM; statistical analysis was with one way ANOVA and Bonferroni comparison of means. Troponin increased post operatively peaking at 6 hours; levels were still significantly elevated compared to baseline at 24 hours (p=0.043, Bonferroni comparison).

Wet: Dry ratio was measured at eight hours (Group 1) and at 24 hours (Group 2), and compared to a sham animal (one who had a normal saline infusion for four hours and then organs harvested). There was no cardiac oedema noted over the 24 hour period:

Sham: 4.5 8 hour: 4.21 +/- 0.25 24 hour: 4.52 +/- 0.14

Results are reported as mean ratio +/- SEM.

Histology:

Histological changes were assessed on H&E stained slides according to the scoring system detailed in Chapter 2. At eight hours, mild to moderate myocyte degeneration but no necrosis was observed. Inflammatory cells were noted. The majority of the slides demonstrated vascular endothelial activation.

By 24 hours, these changes had resolved, with essentially normal histological examination at this time.

This results and representative images are shown below. The table indicates the histological features examined in the heart. Each parameter was graded from 0 to 3 by a blinded pathologist. A composite score was then used to compare the histological specimens at baseline (using the sham animal), 8 hours and 24 hours.

Heart	
Myocytes	
	Necrosis
	Degeneration
Interstitium	
	Inflammatory cells
	Oedema

	Haemorrhage
Pericardium	
	Inflammatory cells
	Oedema
	Haemorrhage
Vessels	
	Endothelial activation
	Obliteration/thrombosis
	Vasculitis

Table 1: Histological features examined in the harvested cardiac tissues.

Composite histological score:

Results are reported as mean +/- SEM. Statistical analysis was with paired t test to compare the 8 hour and 24 hour composite scores.

Baseline	**8 hours**	**24 hours**
0	7 +/- 1.1	1.8 +/- 0.2
	*	*p<0.01

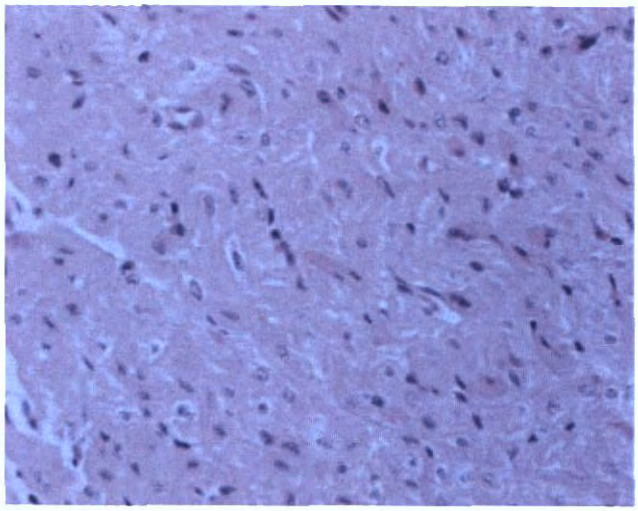

Image 1: Sham Heart demonstrating normal histology

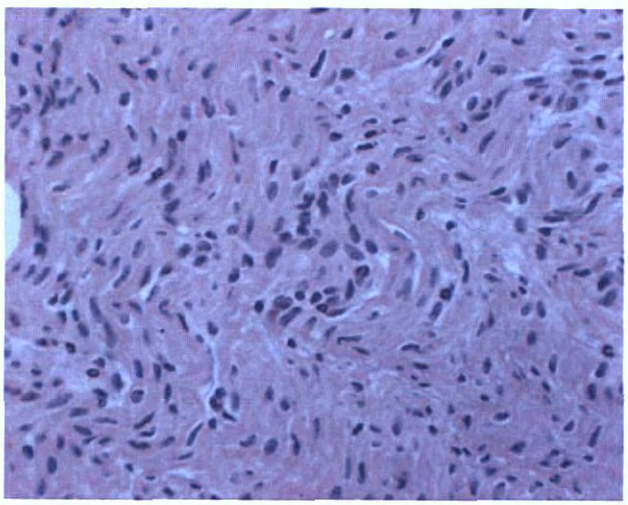

Image 2: Heart at 8 hours – neutrophil infiltration in the tissues and myocyte degeneration were the 2 main features seen.

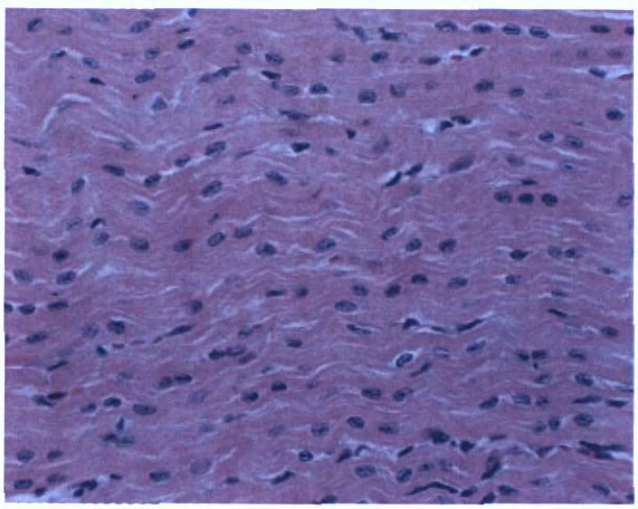

Image 3: Heart at 24 hours – normal histological appearance.

3.3.2 Development of a low cardiac output state

Heart rate, blood pressure, and central venous pressure were recorded hourly. As markers of tissue perfusion, mixed venous oxygen saturations, lactate and base excess were recorded two hourly, and renal and cerebral NIRS hourly. The patterns seen are very interesting. While there was no gross hemodynamic instability as measured by heart rate, mean arterial blood pressure and central venous pressure, there was development of a low cardiac output state as occurs clinically.

In all animals, a tachycardia developed which was significantly increased from baseline at 4 hours. Tachycardia can be attributable to a number of causes in the clinical situation, including pain, inotropes, low cardiac output syndrome, volume depletion as well as SIRS. In our model, none of our animals were on dopamine at 4 hours (all were weaned by 3 hours post reperfusion, many sooner than this). A constant infusion of fentanyl was used for anaesthesia and analgesia; boluses were administered if the piglets were felt to be light on anaesthesia. As the blood pressure and central venous pressure were stable at this time, this tachycardia was felt to be less likely due to volume depletion. In addition, our cardiac catheter readings did not show any deterioration in ventricular function at this time. Therefore, we felt this tachycardia was most likely a direct manifestation of the systemic inflammatory response.

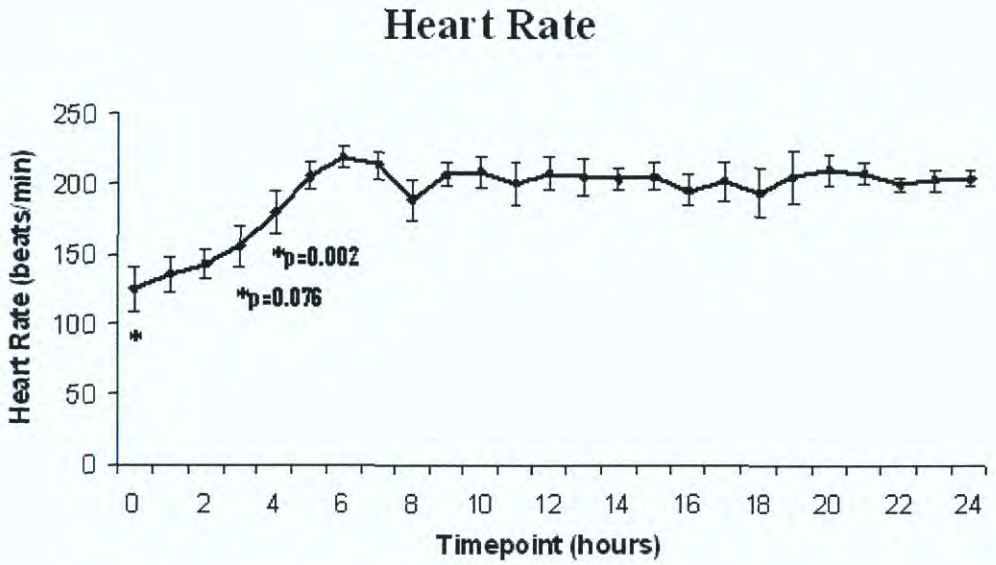

Figure 3.3

Heart rate was recorded hourly. Results are reported as mean heart rate (beats per minute) +/- SEM. Statistical analysis was with one way ANOVA and Bonferroni comparisons. From baseline, a tachycardia develops post-operatively, which was statistically significant from 4 hours onwards and sustained to 24 hours.

Mean arterial blood pressure was recorded hourly. At all times, it was maintained above the critical value of 55 – 60mmHg. However, there was a statistically significant reduction from baseline readings from 8 hours onwards.

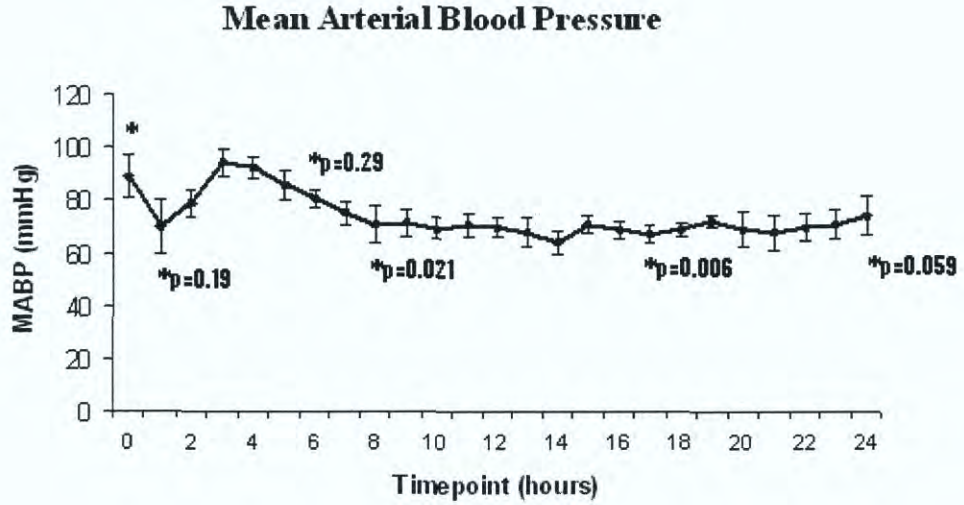

Figure 3.4

Mean arterial blood pressure was recorded hourly from the femoral arterial line. Results are reported as mean MABP (mmHg) +/- SEM. Statistical analysis was with one way ANOVA and Bonferroni comparison of means. The apparent initial drop in MABP at one hour post reperfusion was not statistically significant. MABP trended towards a reduction from baseline values in the early post-operative period and this reduction was statistically significant from 8 hours onwards; however the MABP was always maintained above the critical level of 55 – 60 mmHg. At the 24 hour readings, MABP had returned to baseline (p=0.059, Bonferroni comparison).

Systolic and diastolic blood pressure readings were also analyzed separately in order to determine if one or both were responsible for the reduction in the mean arterial blood pressure value. Systolic blood pressure remained stable throughout. However, diastolic blood pressure demonstrated an early reduction from baseline values which recovered by

two hours post reperfusion. There was then a sustained reduction in diastolic blood pressure from baseline values from 7 hours onwards. It is therefore this reduction in diastolic BP which accounts for the reduction in MABP while systolic blood pressure is maintained.

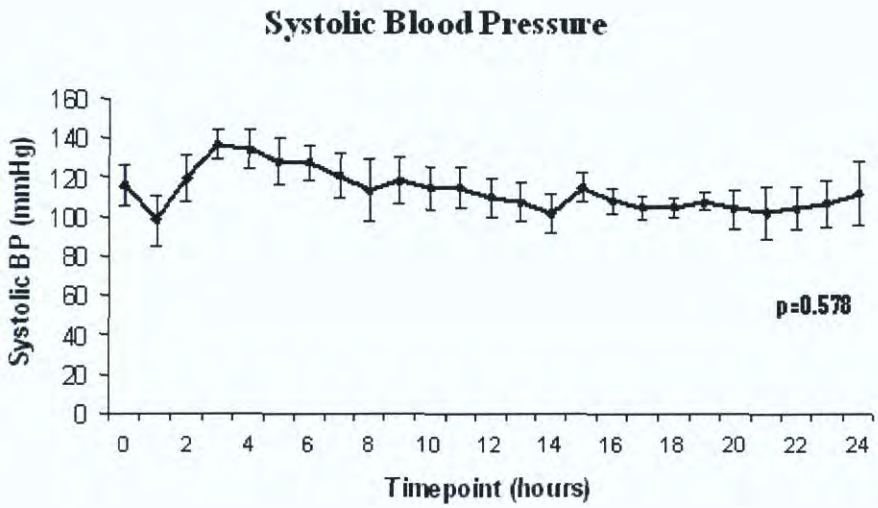

Figure 3.5

Systolic blood pressure was recorded hourly from the femoral arterial line reading. Results are reported as mean systolic blood pressure (mmHg) +/- SEM. Statistical analysis is with one way ANOVA. There was no statistically significant variation in the systolic blood pressure throughout the period of observation (p=0.578, ANOVA).

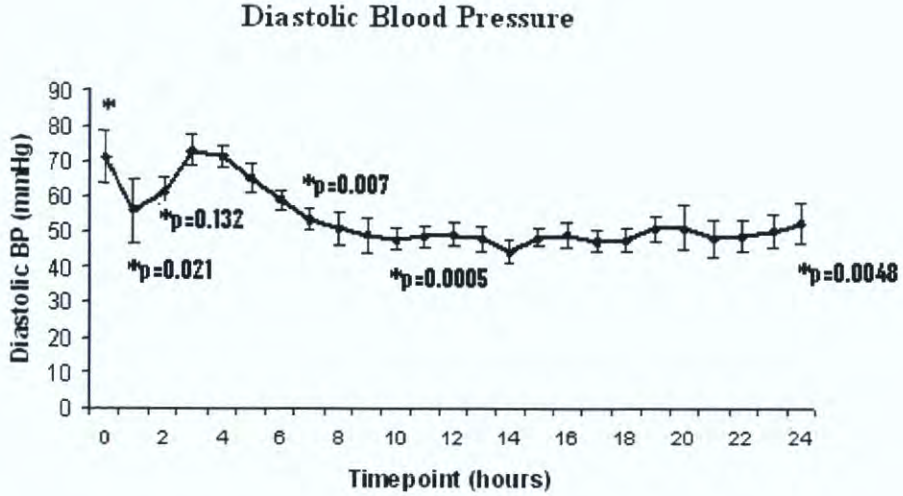

Figure 3.6

Diastolic blood pressure was recorded hourly. Results are reported as mean diastolic blood pressure (mmHg) +/- SEM; statistical analysis was with one way ANOVA and Bonferroni comparison of means. The initial drop in diastolic blood pressure is statistically significant, however this has returned to baseline at two hours post reperfusion. A steady decline is then observed; with the reduction in diastolic blood pressure from baseline becoming statistically significant from 7 hours onwards. This reduction is sustained throughout the remainder of the period of observation.

There was no drop in central venous pressure at any time. In fact, CVP was significantly higher than baseline from 5 hours onwards. Although many factors are important in the determination of adequate intravascular volume, such as right ventricular compliance and diastolic function, CVP is often used as a marker of filling pressures in order to guide volume administration.

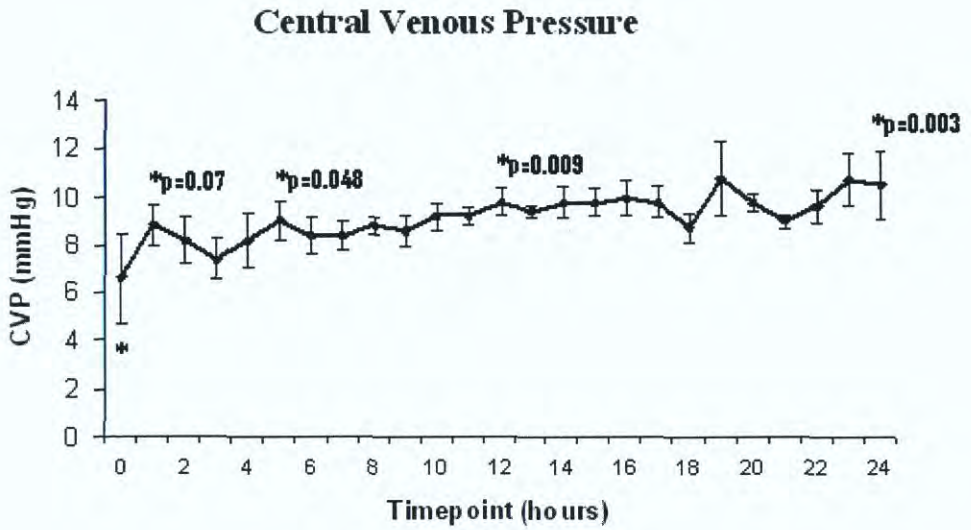

Figure 3.7

Central venous pressure was recorded hourly. Results are reported as mean central venous pressure (mmHg) +/- SEM. Statistical analysis was with one way ANOVA and Bonferroni comparison of means. CVP was adequate at all times.

Mixed venous oxygen saturations showed an initial drop in the first four hours, but then returned to baseline levels and remained stable until a second drop at 24 hours. A reduction in mixed venous oxygen saturations is often used clinically as one of the first indicators of a low cardiac output syndrome. In adults, an MvO_2 of below 50% indicates the onset of anaerobic metabolism, however in neonates, this occurs at 30%. However, due to increased vascular resistance in response to a low cardiac output, the relative contribution of desaturated blood from ischemic regions such as the gut and kidneys is reduced, meaning that ischemia can be occurring in these organs even with a normal

MvO_2 [1] Thus a cut-off point of 65% is employed to indicate a significantly low MvO2 with likely organ ischemia. In this study, the significant drop at 24 hours may be due to the onset of a low cardiac output syndrome. However, given the stability of the rest of our measured parameters at this time, the large drop in MvO2 at 24 hours may also represent an error due to our small numbers.

Lactate and base excess both demonstrated a similar pattern: an initial increase/decrease (a washout phenomenon post bypass), with stable levels then until 20 – 22 hours when a deterioration was again noted.

Regional oxygen saturations also demonstrated an early decrease within the first 3 – 4 hours in both the brain and the kidney. Following a return to baseline levels, renal NIRS then remained stable throughout the remainder of the 24 hours; however, cerebral NIRS demonstrated a second deterioration from 8 hours onwards.

The graphs below display these results.

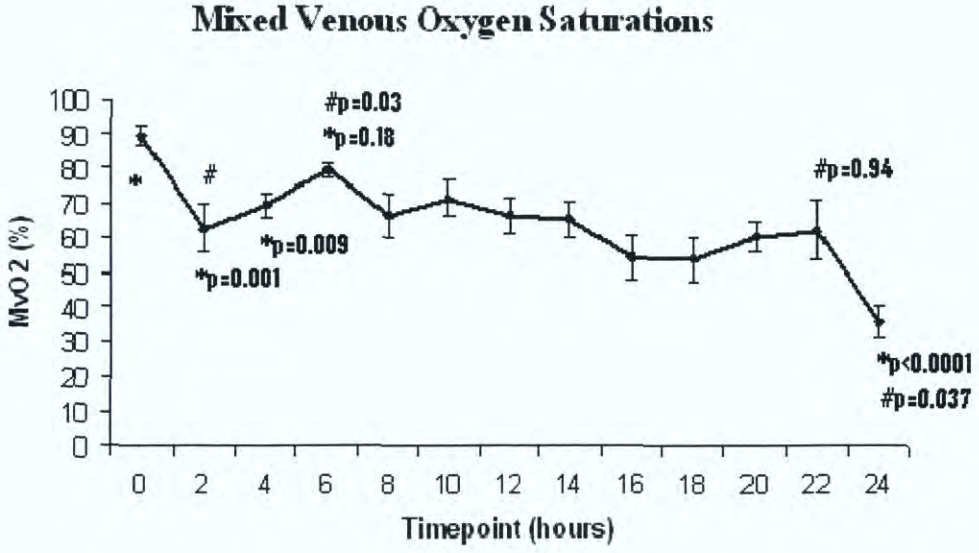

Figure 3.8

Mixed venous oxygen saturations were measured every 2 hours on arterial blood gas analysis. Results are reported as mean mixed venous oxygen saturations (%) +/- SEM. Statistical analysis was with one way ANOVA and Bonferroni comparison of means. MvO2 was significantly reduced from baseline at 2 and 4 hours, but returned to baseline levels at 6 hours. However, from 8 hours onwards, it was again significantly reduced from baseline and remained so for the entire period of observation. Interestingly, on examining the post-operative course, there was a significant improvement in MvO2 from 2 hours to 6 hours (p=0.03, ANOVA), and values then remained stable until 22 hours; a significant drop was then again seen at 24 hours (p=0.04, ANOVA).

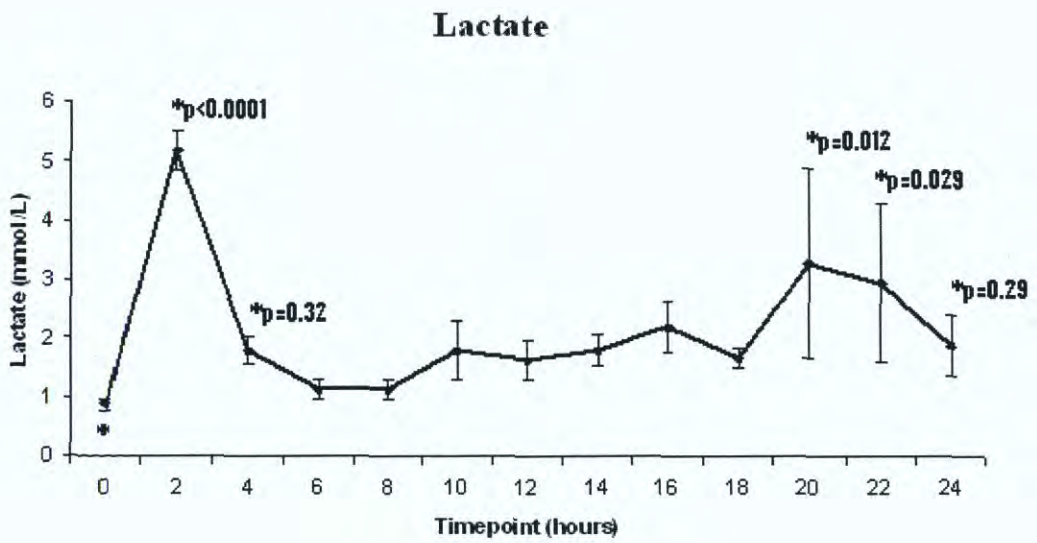

Figure 3.9

Lactate was measured two hourly on arterial blood gas analysis. Results are reported as mean lactate (mmol/L) +/- SEM. Statistical analysis was with one way ANOVA and Bonferroni comparison of means. Lactate showed an initial peak at two hours, a washout phenomenon post bypass; levels returned to baseline values by 4 hours. A second increase was then observed from 18 hours, which was statistically significant at 20 and 22 hours; by 24 hours lactate levels had again returned to baseline values.

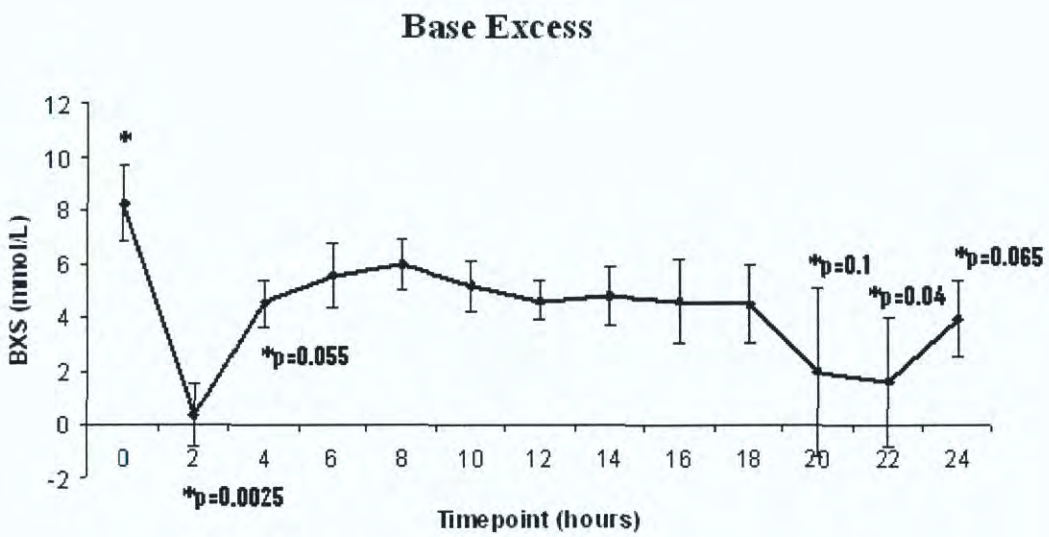

Figure 3.10

Base excess was measured two hourly on arterial blood gas analysis. Results are reported as mean base excess (mmol/L) +/- SEM. Statistical analysis was with one way ANOVA and Bonferroni comparison of means. A similar pattern to lactate levels was observed: an initial washout phenomenon, with stable levels then until a second decrease at 20 – 22 hours.

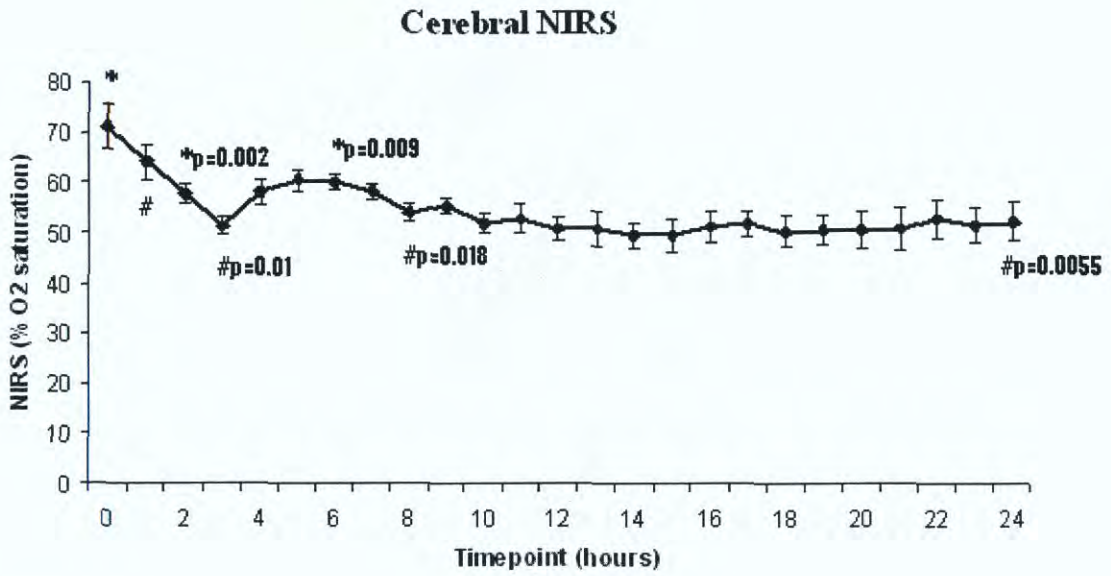

Figure 3.11

Cerebral regional oxygen saturations measured by NIRS were recorded hourly. Results are reported as mean NIRS reading (% oxygen saturation) +/- SEM. Statistical analysis was with one way ANOVA and Bonferroni comparison of means. From 2 hours on, cerebral NIRS readings were significantly reduced from baseline. Looking at the post-operative course, there was a significant reduction in regional oxygen saturations from 1 to 3 hours; the readings were stable from 4 hours to 7 hours. However, at 8 hours, the cerebral NIRS were significantly reduced from the 1 hour reading and remained so for the remainder of the 24 hours.

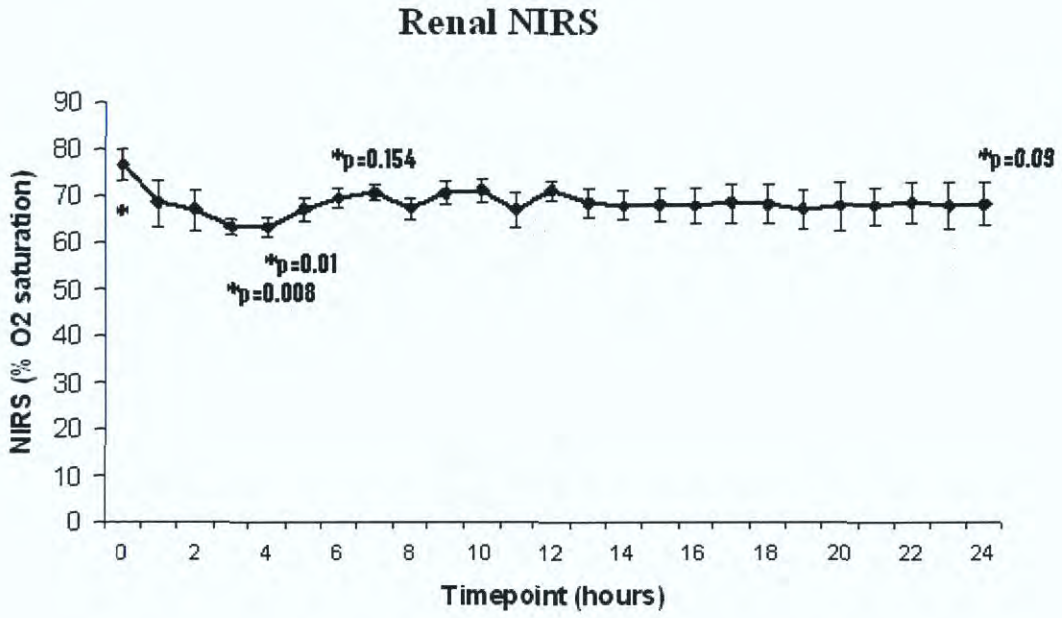

Figure 3.12

Renal regional oxygen saturations were measured by NIRS and recorded hourly. Results are reported as mean NIRS (% oxygen saturation) +/- SEM. Statistical analysis was with one way ANOVA and Bonferroni comparison of means. Renal NIRS decreased from baseline in the early post-operative period; this was *statistically significant* at 3 and 4 hours. However, from 5 hours onwards, there was no difference in the NIRS readings from baseline and they remained stable throughout the remainder of the period of observation.

As factors affecting tissue perfusion and haemodynamic measurements, both nasopharyngeal and core temperatures and also hematocrit were recorded regularly, as shown on the graphs below. Hematocrit remained stable throughout, with a slight

reduction by 24 hours. Temperatures were low initially post-operatively as would be expected, but had returned to baseline by three hours.

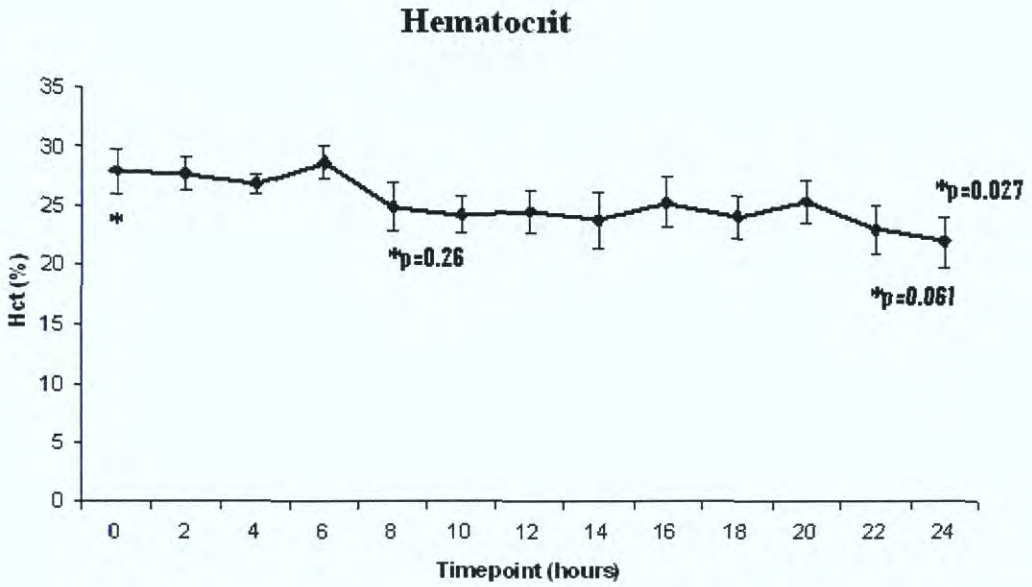

Figure 3.13

***Hematocrit* was recorded from arterial blood gas analysis. Results are reported as mean hematocrit (%) +/- SEM. Statistical analysis was with one way ANOVA and Bonferroni comparison of means. Hematocrit was stable throughout, but was reduced from baseline readings at 24 hours.**

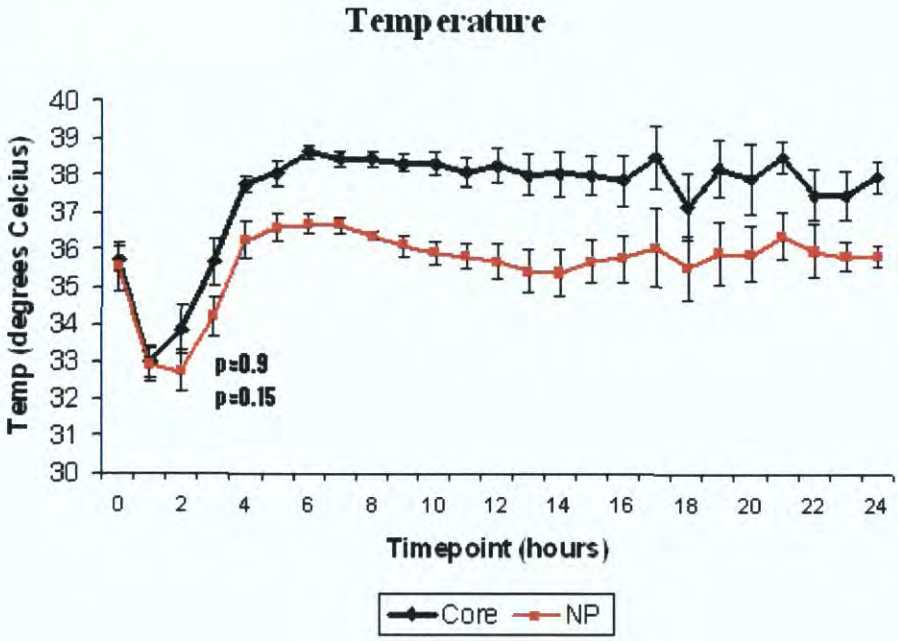

Figure 3.14

Nasopharyngeal and core (rectal) temperatures were recorded hourly. Results are reported as mean temperature (°C) +/- SEM. Statistical analysis was with one way ANOVA and Bonferroni comparison of means. An initial reduction in temperature was evident post operatively, however this had returned to baseline levels by 3 hours (core temperature: p=0.9; nasopharyngeal temperature: p=0.14; ANOVA and Bonferroni comparisons at 3 hours) and was maintained at physiological levels following this.

Correlating all of these cardiac and haemodynamic results, two periods of low cardiac output are noted. The first occurs immediately post reperfusion: a decrease in diastolic blood pressure occurs in the first hour (although MABP is maintained); and a reduction in

mixed venous oxygen saturations, cerebral and renal NIRS is evident until approximately 4 hours. Lactate and base excess are also significantly elevated at this time; however a proportion of this rise must be attributed to a washout phenomenon and vasodilation from rewarming. From 4 – 6 hours, all of these parameters have returned to normal. A tachycardia develops at 4 hours, the first manifestation of the SIRS. Cardiac injury peaks at 6 hours as measured by troponin. From 8 hours onwards, a mild low cardiac output syndrome is observed, which is most likely SIRS related. Diastolic blood pressure drops at 7 hours (indicative of a low systemic vascular resistance as the cardiac catheter readings of ventricular function are stable), with a consequent reduction in mean arterial blood pressure from 8 hours. In addition, cerebral NIRS are reduced from 8 hours. This low cardiac output state does not become severe until later, with a deterioration in lactate and base excess from 18 – 20 hours; and mixed venous oxygen saturations dropping later again at 24 hours, at which time lactate and base excess have returned to normal.

3.3.3 Pulmonary Injury

Pulmonary variables were assessed with the CO_2SMO Plus respiratory profile system and regular arterial blood gas analysis. All the study animals were mechanically ventilated on 100% oxygen in order to standardize results. Significant pulmonary injury was demonstrated post-operatively. It appeared to occur in 2 phases: initial early changes in the mechanics of ventilation with compliance and airway resistance, followed by subsequent changes in gas transfer as seen with PaO_2/FiO_2 ratio and A-a gradients. Airway resistance increased and pulmonary compliance decreased: these changes were

statistically significant from 4 and 6 hours onwards, respectively. The alveolar: arterial gradient also steadily increased post-operatively, however this only reached significance at 22 hours. These changes were associated with a trend towards a reduction in oxygenation as measured by the partial pressure of oxygen, and a reduction of the PO_2:FiO_2 ratio into the acute lung injury range (200 – 300; ARDS is defined as <200).

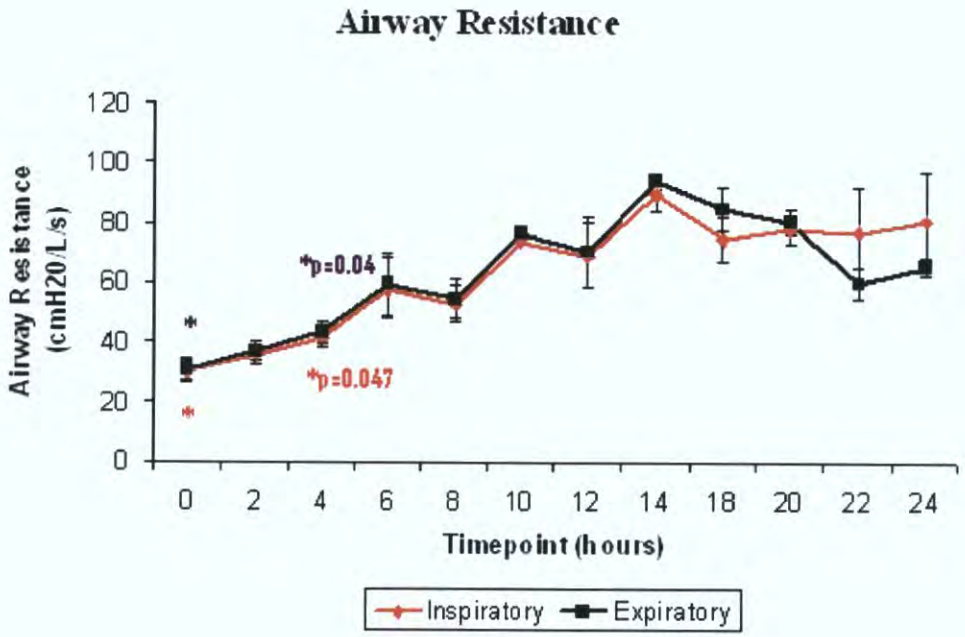

Figure 3.15

Dynamic inspiratory and expiratory airway resistances were recorded every 2 hours from the CO_2SMO Plus respiratory profile system. Results are reported as mean airway resistance (cmH_2O/L/s) +/- SEM. Statistical analysis was with one way ANOVA and Bonferroni comparison of means. Airway resistance steadily increased from baseline; the increase from baseline was significant from 4hours onwards.

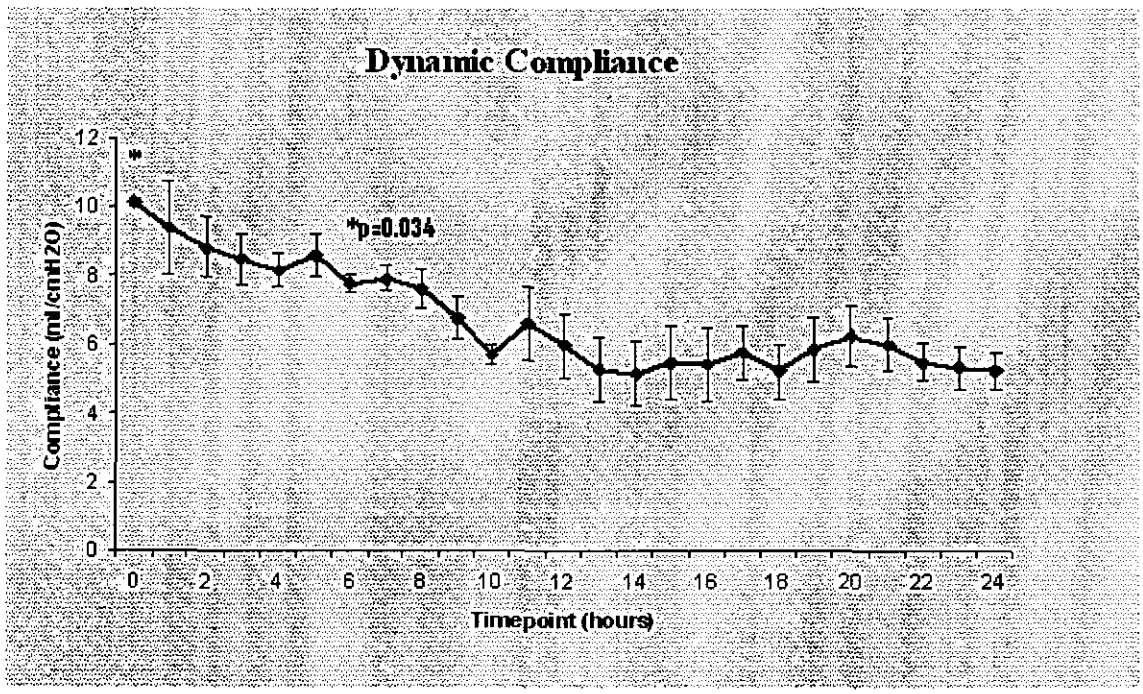

Figure 3.16

Dynamic compliance was recorded hourly using the CO_2SMO Plus respiratory profile monitor. Results are reported as mean compliance (mls/cmH_2O) +/- SEM. Statistical analysis was with one way ANOVA and Bonferroni comparison of means. Compliance steadily decreased from baseline; this reduction was statistically significant from 6 hours onwards.

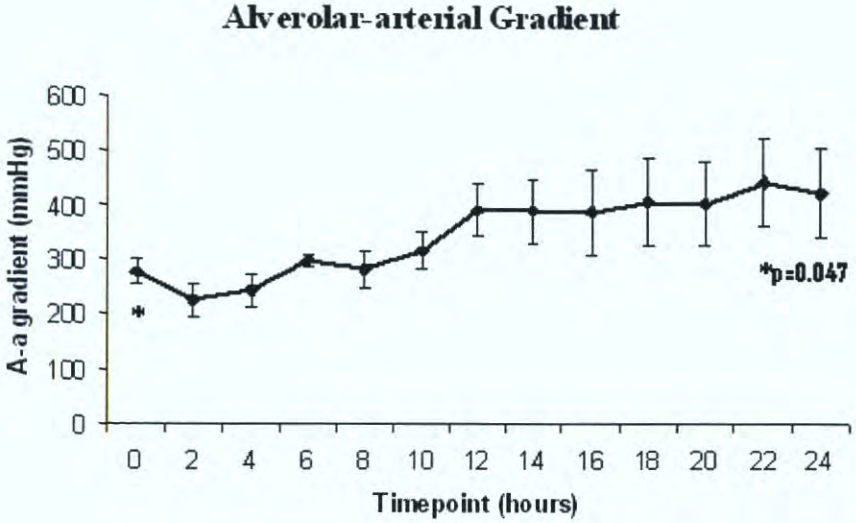

Figure 3.17

Alveolar-arterial gradient ($PAO_2 - PaO_2$) was recorded every two hours. Results are reported as mean A-a gradient (mmHg) +/- SEM. Statistical analysis was with one way ANOVA and Bonferroni comparison of means. A steady increase from baseline levels was seen over the period of observation; this was significant at 22 hours.

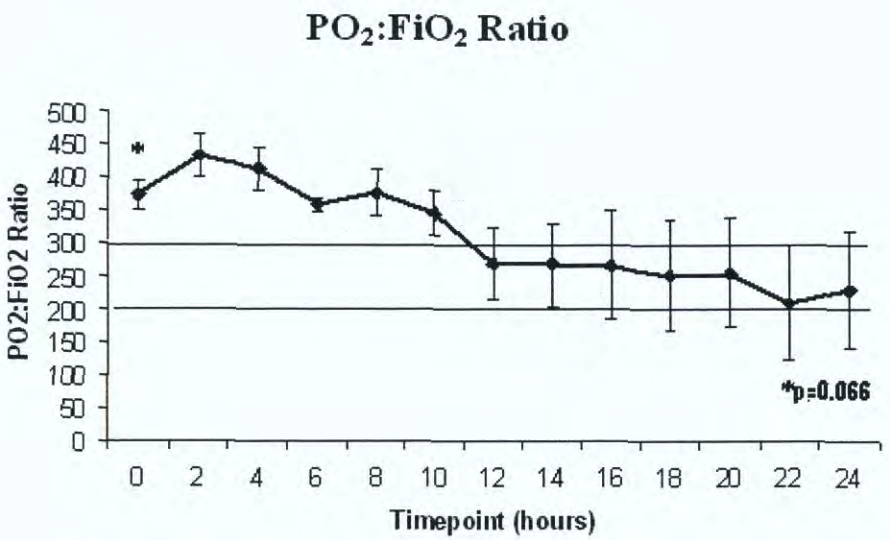

Figure 3.18

PO_2:FiO_2 ratio was assessed every two hours. As all of our piglets were ventilated with 100% O_2, the graphs of PO_2 and the PO_2:FiO_2 ratio show the same results. The pO2:FiO2 graph is shown here. Results are reported as mean PO_2:FiO_2 ratio +/- SEM. Statistical analysis was with one way ANOVA and Bonferroni comparison of means. There was a trend towards a reduction in the PO_2:FiO_2 ratio. Importantly, from 12 hours onwards, the mean values lie in the 200 – 300 range which is defined as acute lung injury (ARDS is defined as <200).

Wet: Dry ratio was measured at 8 hours and at 24 hours and compared to the sham animal. Pulmonary oedema was noted present at 8 hours, with a further elevation at 24 hours:

Sham: 5.1 8 hours: 6.38 +/- 1.49 24 hours: 7.21 +/- 0.45

Results are mean ratio +/- SEM.

Histology:

Histological changes were assessed on H&E stained slides of the harvested lung tissue at 8 hours and 24 hours according to the scoring system detailed in Chapter 2. The factors assessed specifically in the lung are shown in the table below. Each parameter was scored on a scale from 0 to 3 by a blinded pathologist, and a composite score used to compare between the baseline (sham animal), 8 hour and 24 hour results.

Lung	
Alveoli	
	Inflammatory cell infiltration
	Oedema
	Haemorrhage
Interstitium	
	Congestion
	Inflammatory cell infiltration
	Oedema
	Thickening
Pleura	
	Inflammatory cell infiltration
	Oedema
	Haemorrhage
Vessels	
	Endothelial activation
	Obliteration/thrombosis
	Vasculitis

Table 2: Factors assessed on histological examination of the lungs.

At eight hours, inflammatory cells were noted predominantly in the interstitium (moderate amounts) and the alveoli (mild). The interstitium was moderately congested and thickened. Moderate vascular endothelial activation and vasculitis were also seen (it is important to note that this finding is limited by the number of vessels seen on the slide). At 24 hours, this injury had started to resolve, however significant pathology was still observed. The interstitium remained congested and thickened. The inflammatory cells present at this time were macrophages as opposed to the neutrophils observed at 8 hours.

The composite injury scores and representative images of the histology are shown below.

Composite injury score:

Results are reported as mean +/- SEM. Statistical analysis is with paired t test comparing the 8 hour and 24 hour composite scores.

Baseline	**8 hours**	**24 hours**
0	10.6 +/- 0.9	6.8 +/- 2.5
	*	*p=0.16

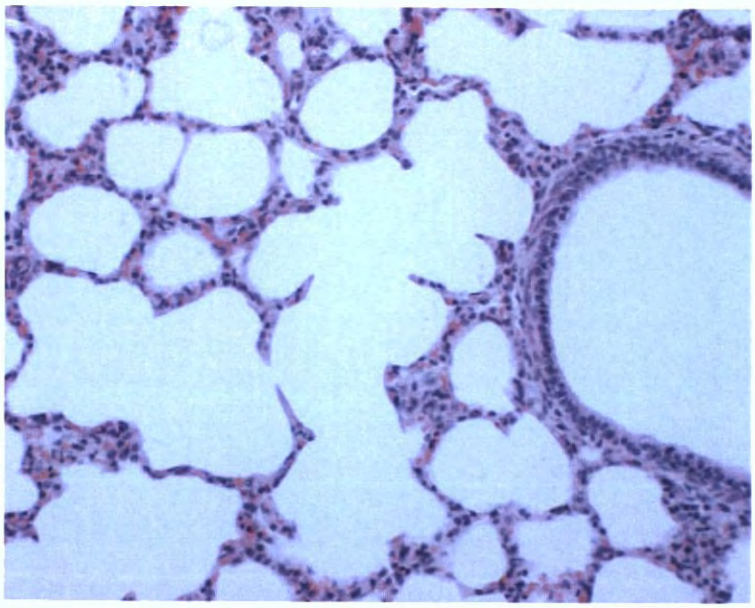

Image 1: Sham Lung demonstrating normal histology

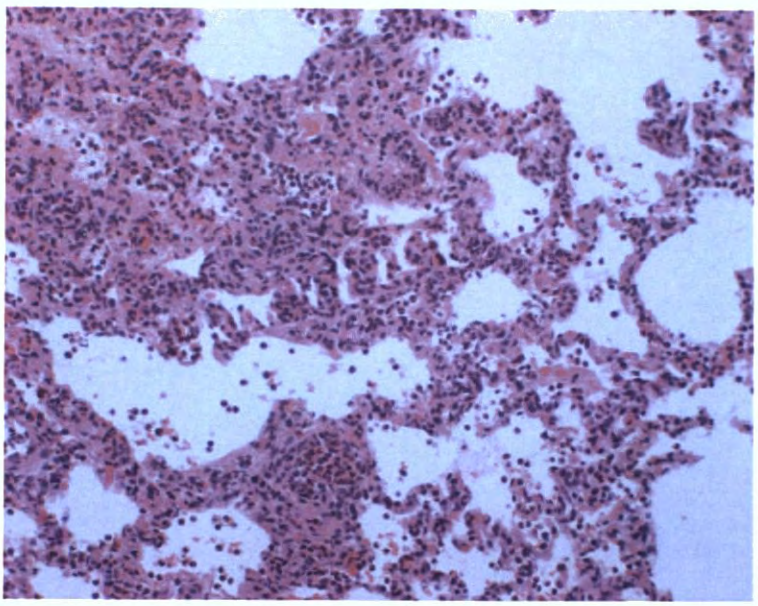

Image 2: Lung at 8 hours – marked thickening of the alveolar septa and significant neutrophil infiltration.

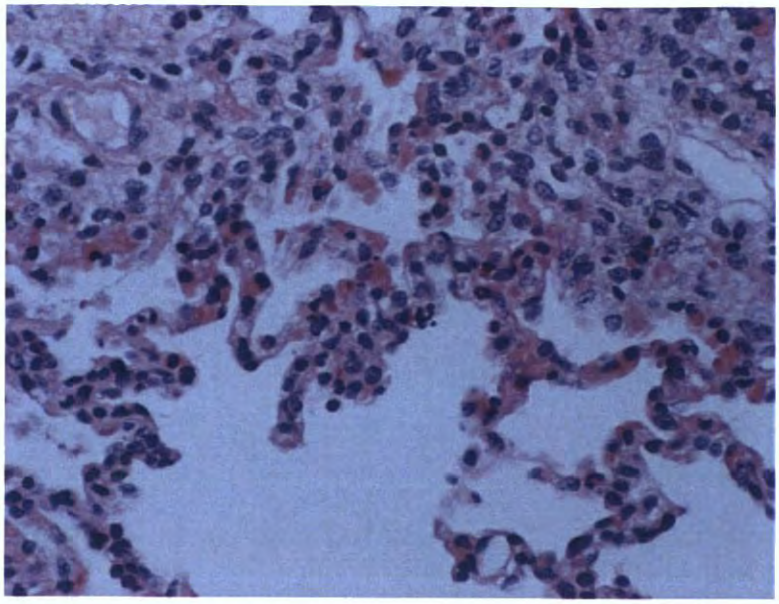

Image 3: Lung at 24 hours – alveolar septal space less thickened than at 8 hours but not yet normal, less marked neutrophilia.

3.3.4 Renal Injury

Renal function was assessed using parameters from each functional area of the kidney, as illustrated on the diagram below.

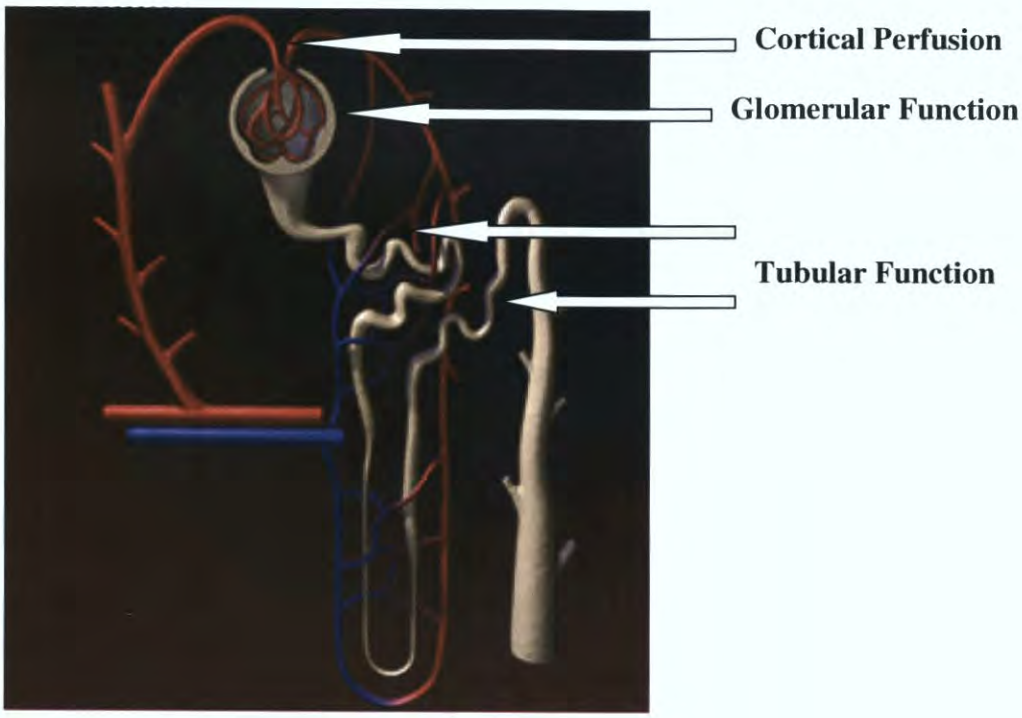

Image 1: The nephron indicating the areas assessed during this study: cortical perfusion (NIRS readings), glomerular function (creatinine clearance), and tubular function (fractional excretion of urinary sodium, and urinary NAG).

A low urine output (<1ml/kg/hr) was evident from five hours onwards. This reduction was preceded by a high urinary NAG level and an increased fractional excretion of urinary sodium at two hours, indicative of tubular structural damage and functional impairment. Glomerular function was not affected, as demonstrated by a normal creatinine clearance at all times. Renal cortical perfusion was maintained as shown by stable NIRS readings at all times.

The renal function patterns are shown in the graphs below.

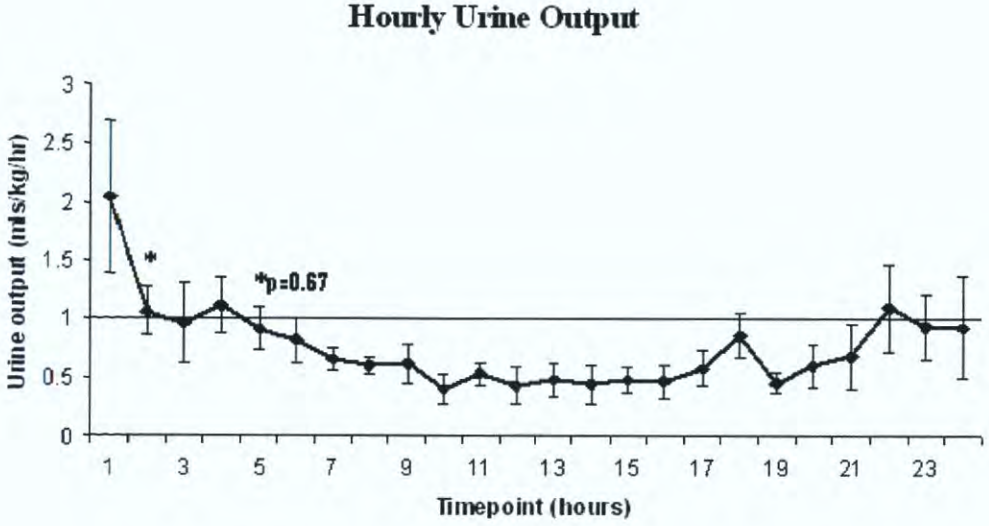

Figure 3.20

Urine output was recorded hourly. Results are reported as mean urine output (mls/kg/hr) +/- SEM. Statistical analysis was with one way ANOVA and Bonferroni comparison of means. There was a trend towards a reduction in urine output, which was recovering towards the end of our period of observation; these changes were not statistically significant when compared to the two hour post reperfusion value. Importantly, urine output was below the critical value of 1ml/kg/hr from 5 hours on.

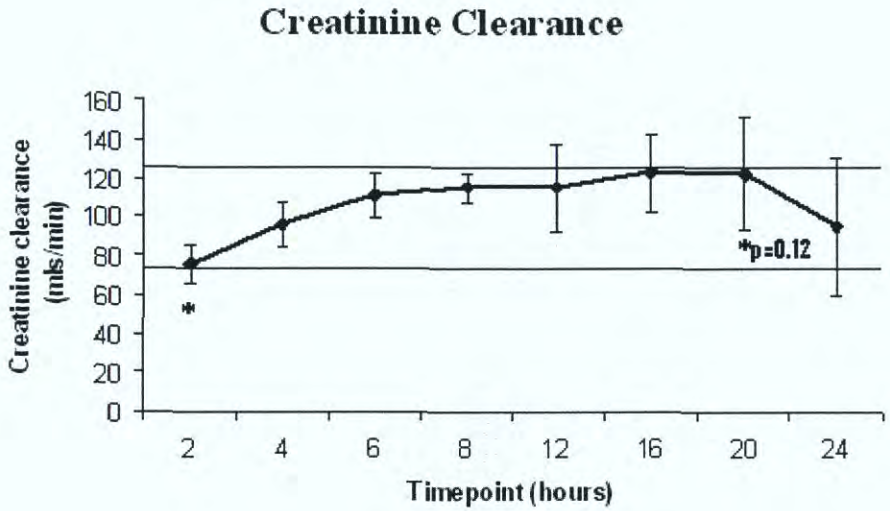

Figure 3.21

Creatinine clearance was measured at the time points shown using the standard formula: CrCl = (urinary creatinine x average hourly urine output per kilogram over the previous two hours x 1.16) / serum creatinine. Results are reported as mean creatinine clearance (mls/min) +/- SEM. Statistical analysis was with one way ANOVA and Bonferroni comparison of means. At all times creatinine clearance remained within the normal range of 75 – 125 mls/min, and there was no significant difference from the two hour value throughout the period of observation.

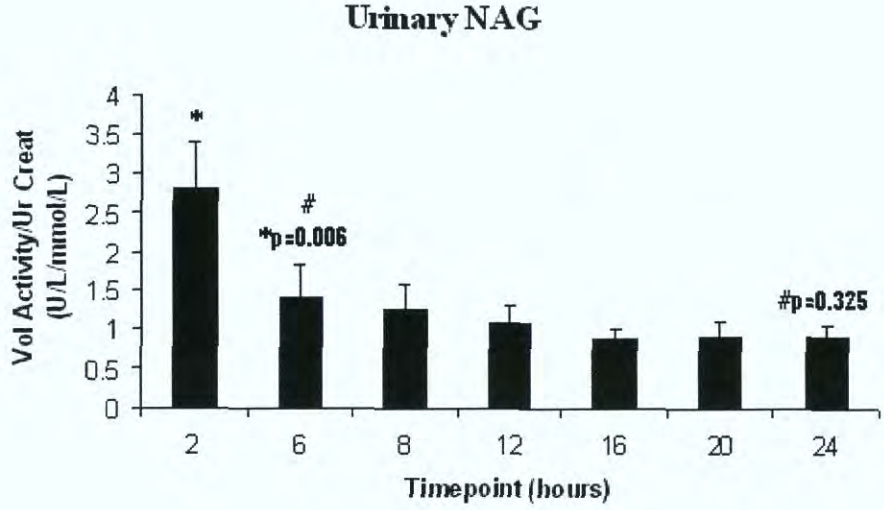

Figure 3.22

Urinary NAG was analyzed at the above time points post reperfusion. Results are reported as mean volume activity of NAG multiplied by 10000 indexed to urinary creatinine (U/L per mmol/L) +/- SEM. Statistical analysis was with one way ANOVA with Bonferroni comparison of means. Levels were high at two hours, but had reduced by six hours and then remained stable to 24 hours.

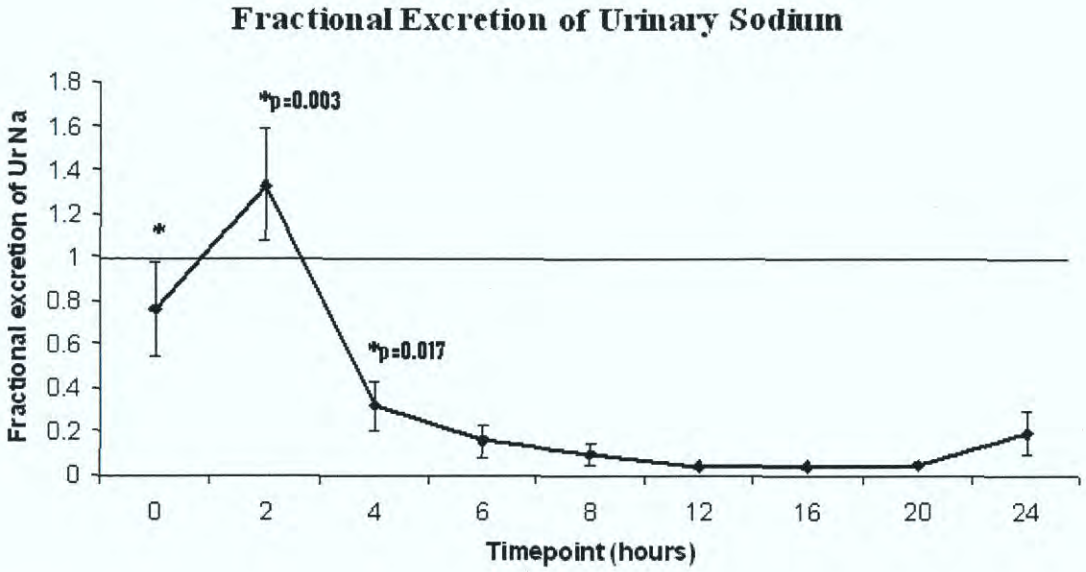

Figure 3.23

Fractional excretion of urinary sodium was recorded at the time points above using the standard formula: {(Urinary sodium/Serum sodium) x (Serum creatinine/Urinary creatinine)} x 100. A level of greater than 1 indicates the loss of the reabsorption capacity of the tubules. Results are reported as mean fraction +/- SEM. Statistical analysis is with one way ANOVA and Bonferroni comparison of means. There is an early significant increase of fractional excretion of urinary sodium above 1, indicative of early tubular necrotic injury.

Wet: Dry ratio was measured at 8 and at 24 hours, and compared to the sham animal. There was no renal oedema noted:

Sham: 5.6 8 hour: 5.78 +/- 0.17 24 hour: 5.503 +/- 0.41

Results are mean ratio +/- SEM.

Histology:

Harvested renal tissue at 8 and 24 hours was assessed according to the scoring system detailed in Chapter 2. The specific factors relating to the assessment of the kidney are shown in the table below. Each was assessed on a 0 to 3 scale of severity by a blinded pathologist and a composite score obtained.

Kidney	
Interstitium	
	Inflammatory cell infiltration
	Oedema
Vessels	
	Endothelial activation
	Obliteration/thrombosis
	Vasculitis
Glomeruli	
	Inflammatory cell infiltration
Tubules	
	Debris
	Cytoplasmic vacuolation

Table 3: Factors assessed histologically on renal specimens.

At eight hours, the interstitium remained essentially normal, with only 2 of 5 slides demonstrating a mild inflammatory cell infiltrate. Moderate vascular endothelial activation was observed, but no vasculitis. In the glomeruli, moderate inflammatory cell infiltrates were observed. Tubular debris was noted in each of the animals, which is important as another sign of tubular necrosis. The severity was mild according to the scoring system employed, however tubular debris will be continually washed away in the

urine produced, therefore the degree seen at histology may underestimate the amount present

Again, as with the heart and lungs, these changes were transient, with a return to a normal histological appearance at 24 hours.

The composite injury scores and representative images are shown below.

Composite injury score:

Results are reported as mean +/- SEM.

Baseline	**8 hours**	**24 hours**
0	5 +/- 1.2	1.8 +/- 0.5
	*	*p=0.04

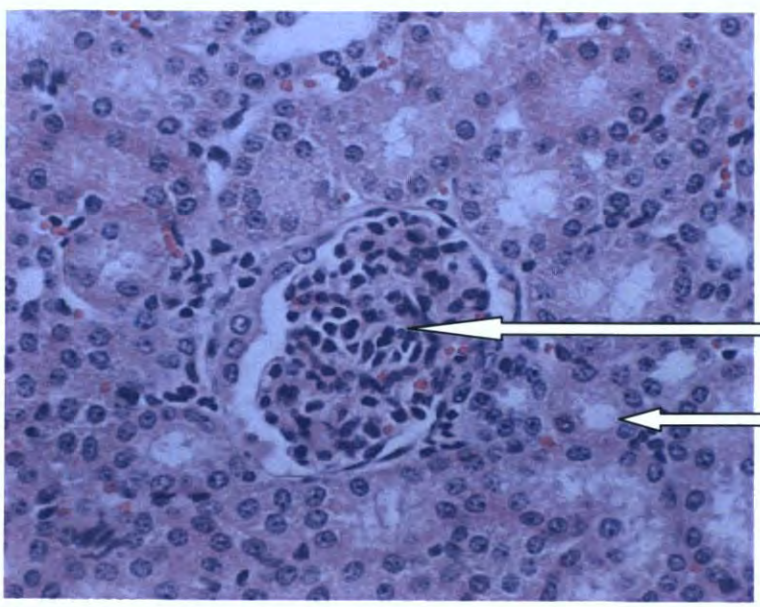

Image 1: Sham Kidney demonstrating normal histology

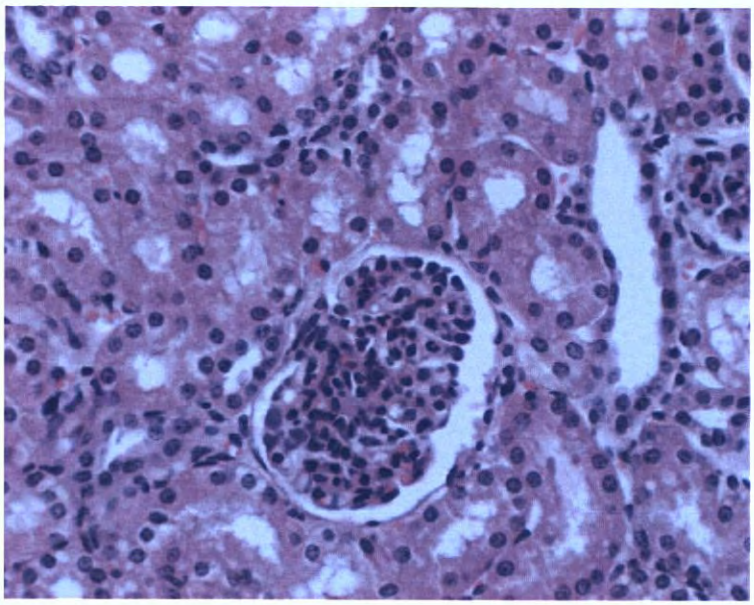

Image 2: Kidney at 8 hours – there is evidence of neutrophil infiltration in the glomerulus and tubular debris.

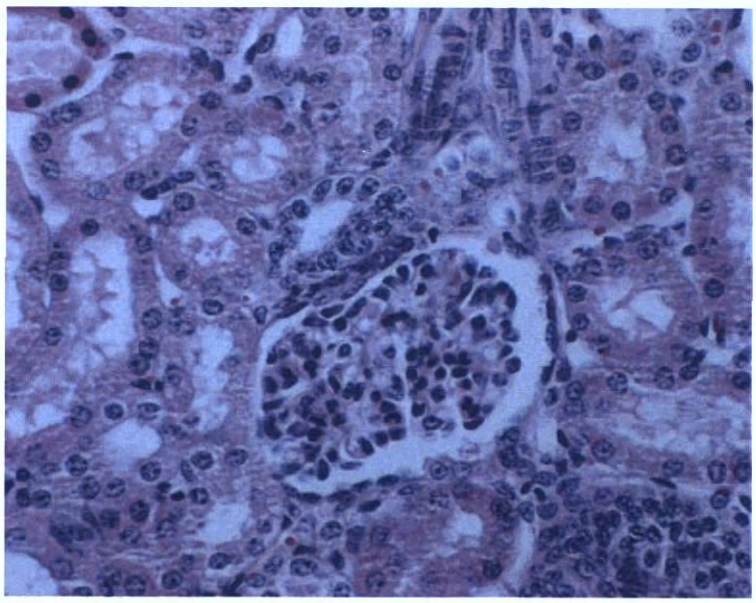

Image 3: Kidney at 24 hours – glomerular neutrophilia resolving, otherwise normal histology.

3.3.5. Mechanism of Injury

White cell counts:

WCC were measured by the Biochemistry laboratory in Beaumont Hospital. An initial early drop immediately post reperfusion was seen – this may indicate sequestration of leucocytes in the tissue, however it is more likely indicative of hemodilution due to bypass. Subsequently, the white cell count rises, secondary to the release of immature granulocytes by the bone marrow, peaking at six hours, and then returns to baseline levels by 18 hours.

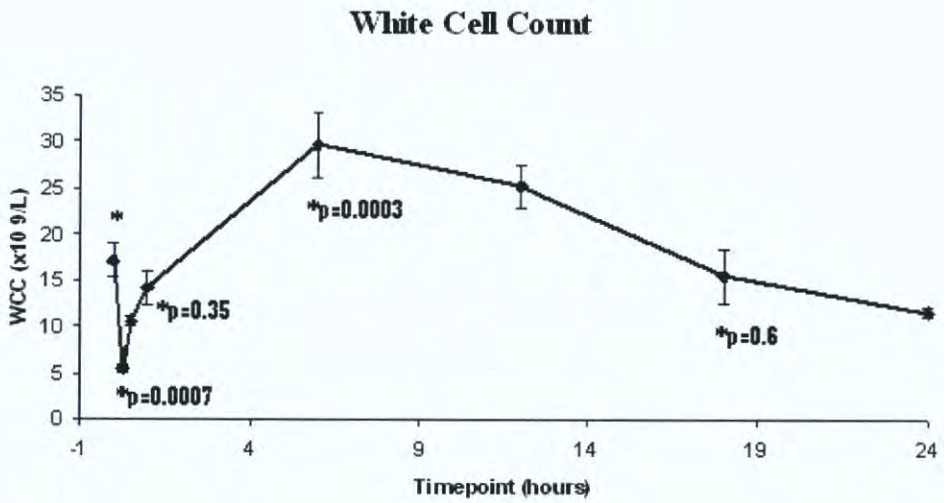

Figure 3.24

White cell counts were measured at the plotted time points. Results are reported as mean white cell count (x10^9/L) +/- SEM. Statistical analysis is with one way ANOVA and Bonferroni comparison of means. In the first hour, WCC dropped significantly

and then returned to baseline levels. It then increased to a peak at 6 hours before returning to baseline levels by 18 hours.

Myeloperoxidase:

Harvested organ samples at 8 hours and at 24 hours were stained using immunohistochemistry for myeloperoxidase, a tissue damaging enzyme released by activated neutrophils in the tissues. The mean counts per 4 high power fields were counted and these results were compared to the sham animal. MPO increased from baseline to 8 hours, most markedly in the lungs, but was returning to baseline levels at 24 hours. A representative image of the lung demonstrating the immunohistological stain is shown below.

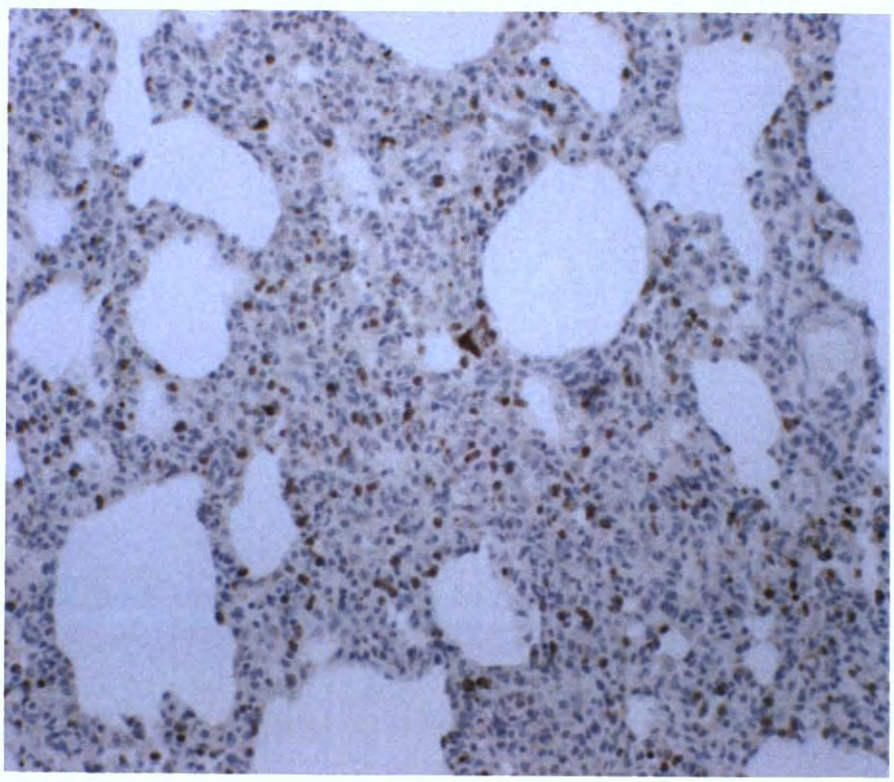

Image 1: Control lung sample at 8 hours showing predominant staining for MPO (brown stains)

The MPO results are demonstrated graphically below.

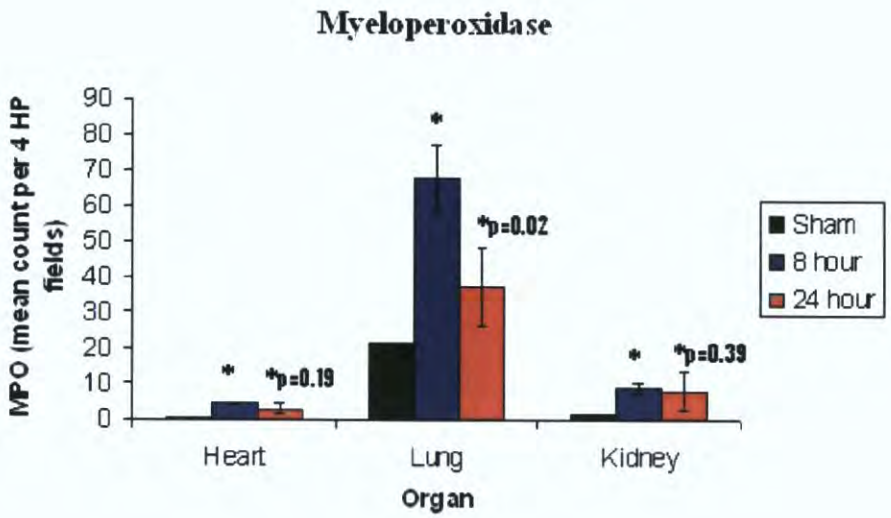

Figure 3.25

Myeloperoxidase was stained in the tissues using immunohistochemistry. Results are reported as mean +/- SEM. Statistical analysis was with paired t test comparing 8 hour and 24 hour values. An increase in MPO levels is seen at 8 hours, most marked in the lung, with return towards baseline levels by 24 hours.

Cytokines:

As markers of the SIRS, levels of the pro-inflammatory cytokines IL-6 and IL-8, and the anti-inflammatory cytokine IL-10 were measured.

IL-8, a potent neutrophil chemotactic factor, was found to be raised on the baseline samples. However, it is important to note that the baseline blood samples are taken

following the four hour infusion and insertion of the femoral arterial and central lines, urinary catheter and endotracheal tube. Therefore this insult alone may have been sufficient to induce IL-8 release. The levels of IL-8 then further increased at two hours post reperfusion (although this was not statistically significant), before decreasing to a low level by 8 hours.

IL-6, the major mediator of the SIRS, increased significantly from baseline peaking at 4 hours. Levels then returned to baseline values by 12 hours.

IL-10, an anti-inflammatory cytokine, followed a similar pattern to IL-6 with a peak increase by 4 hours. However, levels returned to baseline more slowly, being normal at 20 hours.

These trends are illustrated on the following graphs.

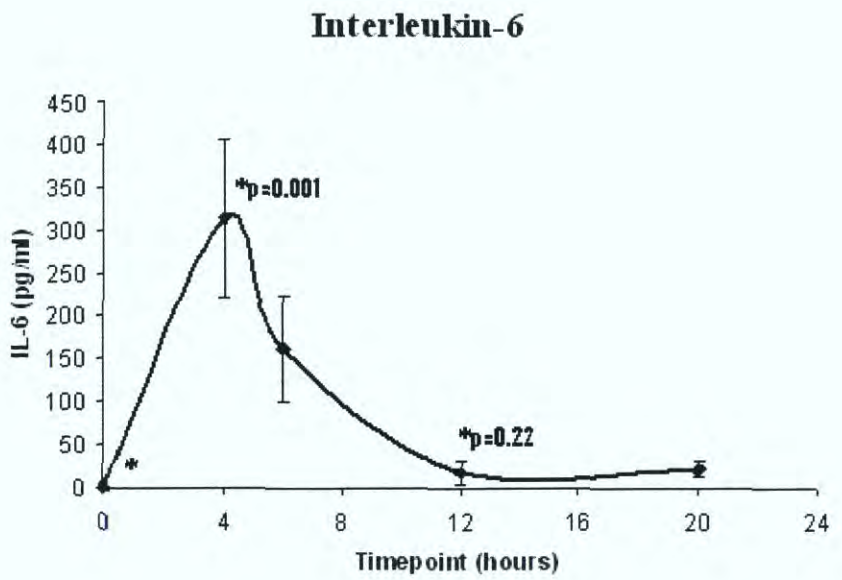

Figure 3.26

Interleukin-6, an important mediator of the acute inflammatory response, was measured with a porcine specific ELISA. Results are reported as mean IL-6 (pg/ml) +/- SEM. Statistical analysis was with one way ANOVA and Bonferroni comparison of means. IL-6 increased from baseline, peaking at four hours and returning to normal levels by 12 hours.

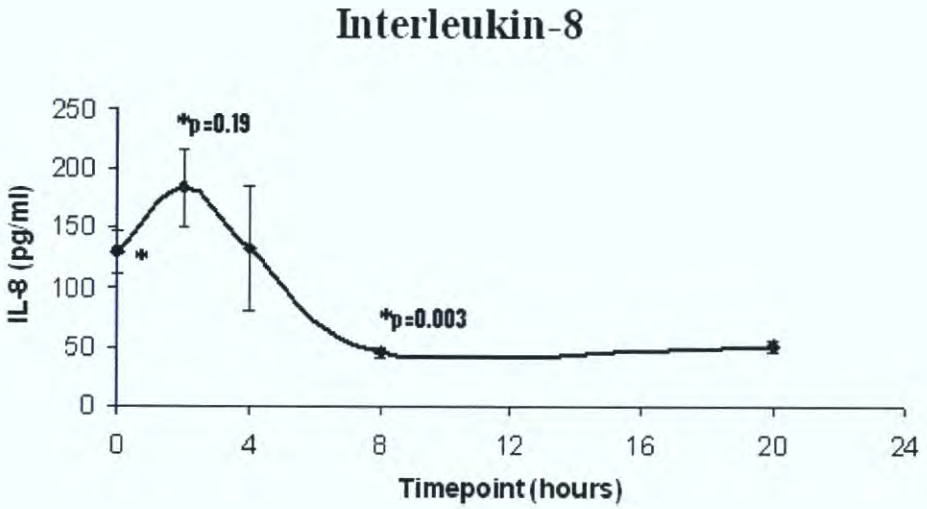

Figure 3.27

Interleukin-8, a potent neutrophil chemotactic factor, was measured using a porcine specific ELISA. Results are reported as mean IL-8 level (pg/ml) +/- SEM; statistical analysis was with one way ANOVA and Bonferroni comparison of means. IL-8 levels were elevated on our baseline sample and did trend towards a further increase at two hour post reperfusion, although this was not statistically significant. Levels then decreased, being lower at 8 hours than at baseline, and remaining stable after that time.

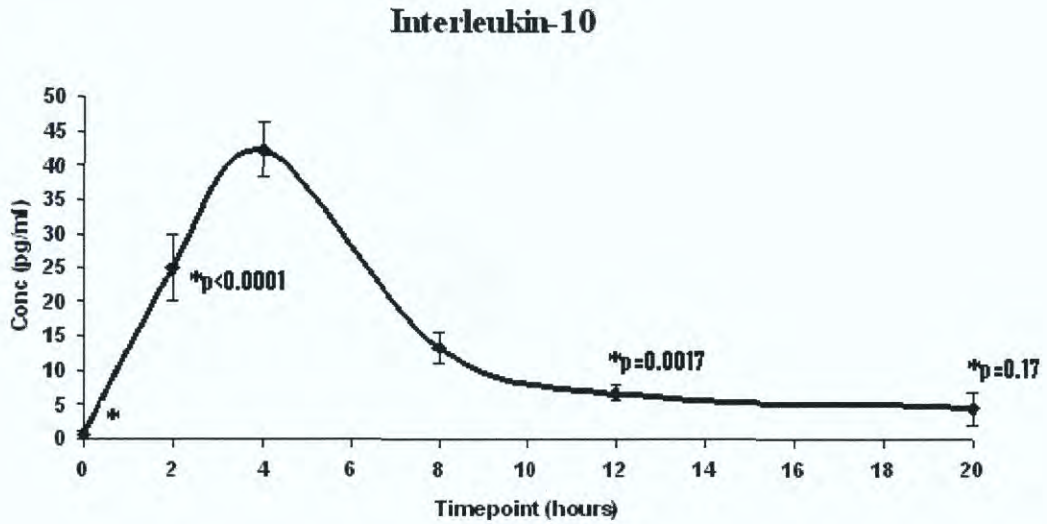

Figure 3.28

Interleukin-10, an anti-inflammatory cytokine, was measured on serum samples using a specific porcine ELISA. Results are reported as mean IL-10 level (pg/ml) +/- SEM; statistical analysis was with one way ANOVA and Bonferroni comparison of means. IL-10 levels were significantly elevated by 2 hours post reperfusion, peaking at 4 hours, and then steadily returning to baseline levels by 20 hours.

Nuclear Factor kappa B (NFkB):

NFkB is a cellular transcription factor which is activated by cytokines such as IL-6 and IL-8 and inhibited by IL-10. NFkB is usually present in an inactive state in the cytoplasm. Once activated, it is then translocated into the nucleus where it up-regulates key endothelial inflammatory response genes. Levels of the active p65 subunit of NFkB were measured in all the major organs at 8 hours and at 24 hours, and compared to levels

at baseline in the sham animal. Our results are shown in the graph below: levels were reduced in all organs at 8 hours; in the lung, the levels were lower again at 24 hours; while in the heart and kidney, levels increased from 8 to 24 hours. The lower levels at 8 and 24 hours demonstrate the relative immune suppressed state that occurs post-operatively.

It is important to note that we extracted a whole cell lysate from our tissue samples; this would predominantly measure the cytoplasmic rather than the nuclear levels of NFkB. In addition, with activation and binding of NFkB in the nucleus, inhibitory kB is also newly synthesized, leading to a negative feedback loop. Thus the rise in NFkB levels in the inflammatory response is usually rapid and transient; therefore, in measuring the samples at 8 hours we may have missed the acute increase.

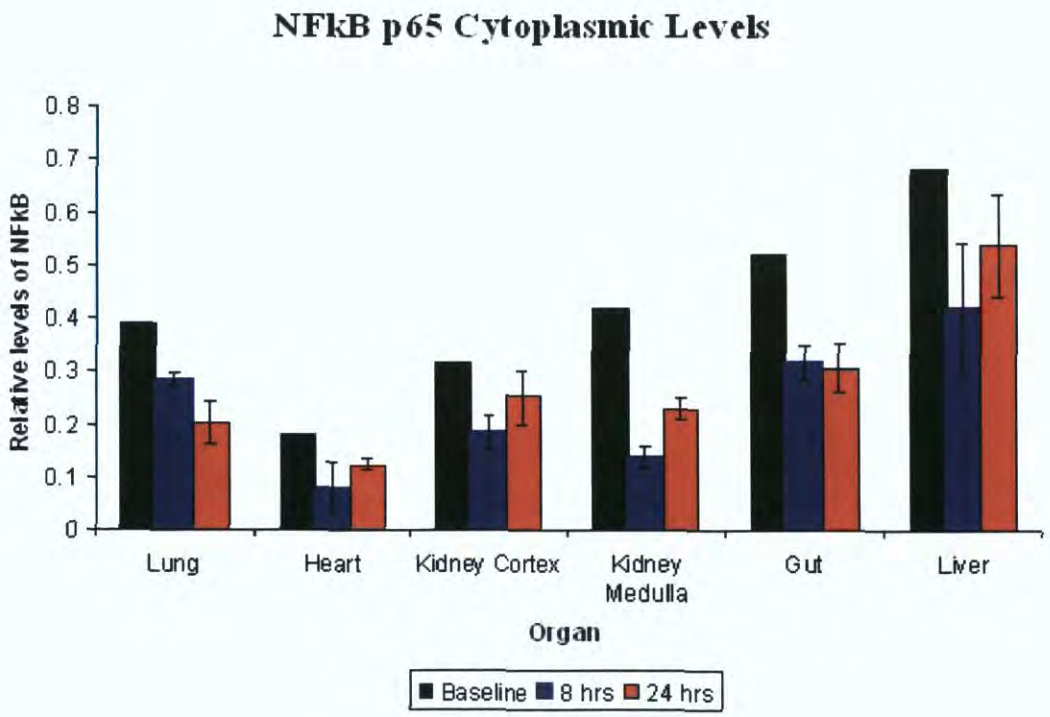

Figure 3.29

NFkB levels were measured at 8 and 24 hours. Results are reported as mean relative levels of NFkB +/- SEM. Levels were reduced from baseline in all organs at 8 hours. At 24 hours, levels in the lung had decreased further, while levels in the heart and kidney had increased compared to the 8 hour values.

3.4 Discussion

A low cardiac output syndrome (LCOS) complicates the post-operative course of 25% of paediatric cardiac surgical patients[2]. The time course varies, but it usually occurs in the first 24 hours, with an onset most commonly at 4 – 6 hours post-operatively[1,3].A study by Wernovsky et al of 170 patients post arterial switch operation for transposition of the great arteries, showed that cardiac index reached a nadir at 9 -12 hours post-operatively returning to baseline values by 24 hours; however, in 9.8% of patients the lowest cardiac index was measured as early as three hours post-operatively[4]. A recent animal study using the piglet cardiopulmonary bypass model also demonstrated very early reduced cardiac output, from 2 – 4 hours post cardiopulmonary bypass[5]. The LCOS is the cause of significant morbidity and mortality, being associated with prolonged mechanical ventilation, longer ICU stays and increased risk of sepsis[4]. It is characterized by reduced perfusion and oxygen delivery to the tissues, manifesting clinically as tachycardia and systemic hypotension, cool peripheries, oliguria, widened arterial-mixed venous oxygen difference and metabolic acidosis[6]. As cardiac output is determined by the contractile function of the heart, the heart rate, the ventricular load and vascular resistance, a low

cardiac output state can therefore be due to one or more of these factors. The exact aetiology of the post-operative LCOS remains debated. Following cardiac surgery, studies have demonstrated impaired ventricular contractility, worst at 4 – 6 hours post separation from cardiopulmonary bypass, with recovery by 24 hours post-operatively[7]. Increased left ventricular afterload, and increased systemic and pulmonary vascular resistance have also been demonstrated in various studies, all of which may contribute to, or be compensatory for, a LCOS[1-4]. Other studies have found low systemic vascular resistance post-operatively secondary to the vasomotor disturbances induced by the SIRS[8,9]. These changes have been attributed to myocardial operative trauma, ischemia-reperfusion injury and SIRS. Many studies have demonstrated associations of mediators of the SIRS with LCOS – for example, IL-8 4 hours post-operatively has been shown to be an independent predictor of LCOS[10]; while higher levels of IL-10 (an anti-inflammatory cytokine) are associated with a normal post-operative cardiac index[11]. This study demonstrated two periods of low cardiac output. The first occurred immediately post reperfusion and lasted until approximately 4 hours. The cardiac catheter readings did not show a decrease in ventricular function at this time, in fact systolic function was increased in the first eight hours (importantly, all the study animals were on a dopamine infusion for the first three hours post separation from bypass). This was unexpected, as the literature indicates that ventricular dysfunction post-operatively is common, and early low cardiac output is thought to be as a result of myocardial stunning[12]. Our results in this regard may be limited by our numbers. Another explanation is an increased peripheral resistance secondary to low temperatures which had returned to normal by 3 - 4 hours post-operatively. The SIRS was observed from 4 –

6 hours: a tachycardia developed, IL-6 and white cell count levels peaked. Also at this time, cardiac injury peaked as measured by troponin. Following this, from 8 hours onwards, a second period of low cardiac output was observed. Diastolic blood pressure dropped at 7 hours (indicative of a low systemic vascular resistance as the cardiac catheter readings of ventricular function are stable), with a consequent reduction in mean arterial blood pressure from 8 hours. In addition, cerebral NIRS are reduced from 8 hours. This low cardiac output state does not become severe until later, with lactate rising significantly at 22 hours and mixed venous oxygen saturations dropping at 24 hours. It is therefore important to note that the cerebral NIRS were the earliest indicator of the LCOS. In the absence of measured ventricular dysfunction in the study animals, and with the observed induction of the SIRS, the impression from this study is that the low cardiac output is likely due to endothelial dysfunction with consequent peripheral vasodilatation and a low systemic vascular resistance. Supporting this was the observation that the animals developed whole body oedema during the study, and required multiple fluid boluses to maintain their mean arterial blood pressure. However, it is possible that our small numbers did not pick up on ventricular dysfunction – in the majority of studies, ventricular dysfunction has been noted and is a well recognized factor in the development of a low cardiac output syndrome.

Pulmonary dysfunction post cardiopulmonary bypass is common, but frequently mild or sub-clinical. In a recent study of 400 adult cardiac surgical patients by Gott et al, significant reductions in compliance and alveolar-arterial gradients were noted post-operatively; however, the majority of these patients did not develop significant

pulmonary complications[13]. ARDS, the most severe form of post-operative pulmonary dysfunction, occurs in 0.5 – 1.7% of post operative patients, and does have a very high mortality[14], particularly in children[15]. The pulmonary dysfunction following CPB is now predominantly attributed to the ischemia-reperfusion injury and SIRS[13]. As the lung is the only organ receiving the whole of the cardiac output, it is therefore exposed to high levels of inflammatory mediators produced by the myocardium[16]. Several studies have demonstrated the association of SIRS with acute lung injury, and that modulation of this response leads to improved post operative respiratory function[13,17,18]. Importantly, a recent animal study showed that lung ischemia-reperfusion causes more severe lung damage in infants than in adults[14], making this complication of particular importance in the paediatric setting.

This study demonstrated significant pulmonary dysfunction post-operatively with significantly increased airway resistance and decreased pulmonary compliance from 4 - 6 hours onwards. These changes were temporally associated with the induction of the SIRS, as shown by increased peripheral white cell count and serum IL-6 at this time, and also with marked pulmonary oedema at eight hours, which is most likely secondary to the inflammatory vascular endothelial dysfunction. In addition, histology at eight hours showed marked inflammatory cell infiltration in the tissues; this was confirmed by myeloperoxidase measurement. Following these changes, the alveolar: arterial gradient, a measure of alveolar-capillary gas exchange, steadily increased and reduced oxygenation, as measured by the partial pressure of oxygen and a deterioration of the

PO_2:FiO_2 ratio into the acute lung injury range (200 – 300), was observed. Interestingly, there was still significant histological injury and oedema at 24 hours.

Acute renal dysfunction following cardiac surgery is a significant cause of morbidity and mortality, both in adult and paediatric populations. The pathophysiology of this injury is extremely complex, and all aspects are hotly debated. This study showed a proximal tubular structural injury, as evidenced by an increase in urinary NAG levels; and a distal tubular functional impairment, as measured by the fractional excretion of urinary sodium. This injury occurred early, peaking at two hours. This pattern is in keeping with previous studies in the literature[19,20,21]. Creatinine clearance, as a measure of glomerular function, remained normal throughout our study. Renal cortical NIRS was also normal indicating normal renal cortical perfusion. Therefore the injury we observed, as evidenced by the biochemical markers and the reduction in urinary output, was tubular in nature, rather than glomerular, and it occurred early. The literature on the whole does point to acute tubular necrosis as the pathology seen in acute renal injury following cardiac surgery[22]. This is evidenced by experimental models of hypoxia, and many studies of specific renal biochemical markers. In addition, in the early stages of ATN, there is evidence for both proximal and distal tubular injury, as measured by specific markers, which would support our findings[23]. However, some studies have shown glomerular capillary damage as measured by microalbuminuria[16] and other specific glomerular markers of injury (albumin, transferrin and immunoglobulin G)[24]; and reversible alterations in the structure of the glomeruli during hypothermic cardiopulmonary bypass have also been

demonstrated[25]. The limitation of this study is that only creatinine clearance was used as the marker of glomerular function.

With regard to the cause of the renal injury seen post cardiac surgery, again multiple theories abound. Ischemia is clearly important. This is particularly important in the medulla (the location of the renal tubules) as it is supplied by the efferent arterioles and is therefore more susceptible to hypotension. Our renal NIRS readings were significantly reduced from baseline at three and four hours post reperfusion, however at this time the injury has already occurred being most evident at two hours. Importantly, although a low cardiac output state develops in our model later from 10 – 12 hours onwards, renal function does not appear to worsen with this. It is likely therefore be that the renal injury is due to an intra-operative insult. A number of factors contribute to renal hypo-perfusion peri-operatively, including haemodynamic instability, volume depletion and the non pulsatile flow of cardiopulmonary bypass which induces renal vasoconstriction. Pulsatile perfusion in an animal model has been shown to increase renal blood flow intra-operatively and in the early post-operative period[26], and a study using biologically variable perfusion also demonstrated a reduction in the excretion of urinary enzyme markers of tubular injury post operatively[27]. In addition, avoidance of cardiopulmonary bypass with off-pump surgery has been shown to improve renal function post-operatively[28,29]. This may be due to the avoidance of non-pulsatile flow.

Off-pump surgery may also be beneficial in terms of renal function due to the avoidance of the bypass circuit and the consequent inflammatory response, which is a second

possible cause of the renal injury following cardiac surgery. A recent study of 62 CABG patients demonstrated a significant association of increased IL-6 production with acute renal dysfunction (defined by increases in blood creatinine) post-operatively[18]. In addition, a study looking at IL-8 and TNF-alpha in renal function post cardiac surgery demonstrated that both of these pro-inflammatory cytokines correlated with proximal tubular injury as measured by the urinary NAG/creatinine ratio[30]. However, these studies demonstrated associations rather that cause and effect. It has been shown that the kidney preferentially filters the smaller pro-inflammatory cytokines rather than the larger anti-inflammatory ones. This is thought to cause proximal tubular damage via the production of nitric oxide (NO)[17,18,31]. However, studies with steroids and leucocyte depletion following cardiac surgery have demonstrated conflicting results in reducing renal injury[21]. In our study, peak cytokine levels were shown at approximately 4 hours following reperfusion, followed by peak peripheral white cell counts at 6 hours. Thus, the early tubular injury observed is occurring prior to the onset of the SIRS and in addition, renal function is not worsened by the SIRS. Thus this is unlikely to be the cause of the pathology observed.

Another theory for the renal injury seen post-operatively is the increased activity of the renin-angiotensin system during cardiopulmonary bypass. Plasma renin activity has been shown to increase significantly during CPB and in the first six hours post CPB[32]. Angiotensin II (a potent vasoconstrictor) has also been shown to be increase during CPB and correlates with an increase in peripheral vascular resistance[33]. With regard to the renal effects of this system, a recent study observed a reduced rate of acute renal injury in

CABG patients who were taking angiotensin converting enzyme inhibitors pre-operatively[34]. Also, intravenous enalapril administered during surgery with CBP to patients with altered left ventricular ejection fraction improved renal perfusion both during the surgery and up to seven days post-operatively; importantly this did not cause any significant changes in blood pressure[35]. However, an interesting study comparing modes of perfusion demonstrated that although pulsatile perfusion abolished the rise in plasma renin levels seen in non-pulsaile flow, there was no significant difference in the urinary excretion of beta-2 microglobulin (a marker of proximal tubular dysfunction)[29]. Thus, the contribution of this vasoactive pathway to renal injury remains unclear, and it would be interesting to investigate it further in our model by measuring the levels of renin and angiotensin.

The findings in this study in relation to the SIRS correlate well with previous studies. IL-8 peaked early at 2 hours as expected[36]. IL-8 is produced both by macrophages via gene transcription, but also can be released rapidly from endothelial cell specific storage granules, the Weibel-Palade bodies[37], thus accounting for its early rise in this study. IL-6, which is produced predominantly by macrophages, peaked at 4 hours in our study; again this is consistent with the literature[38].

Nuclear factor kappa B (NFkB) is usually present in the cytoplasm of cells bound to its inhibitory protein IkB. When stimulated (such as following CPB[39]), IkB dissociates and NFkB is translocated to the nucleus where it induces the transcription of many pro-inflammatory genes, including TNF-alpha, IL-6, IL-8, ICAM-1 and E-selectin[40,41]. However, as with many systems in nature, once in the nucleus it initiates a self regulatory

feedback loop by the induction of the production of new IkB. In addition, IL-10 is an inhibitor of NFkB[42]. An increase in NFkB would have been expected due to ischemia-reperfusion and the induction of the SIRS due to CBP. However, the opposite was in fact demonstrated - lower levels at 8 hours. This is most likely due to the negative feedback loop and the influence of IL-10, which peaked at 4 hours, therefore the period of increased levels of NFkB was possibly missed. In addition, the technique used to measure NFkB using whole cell lysate of organ tissue will have measured predominantly cytoplasmic rather than nuclear levels of NFkB; thus the activated levels may in fact be higher.

Therefore, to summarize, this study has shown significant early cardiac, pulmonary and renal dysfunction in a model of juvenile piglet cardiopulmonary bypass and circulatory arrest. Induction of the SIRS has also been demonstrated, which can be related in time to the cardiopulmonary injury. Pulmonary injury is almost exclusively related to the development of SIRS and is still significant at 24 hours. The renal injury observed is purely tubular and occurs very early post reperfusion with subsequent recovery. This model did not demonstrate any correlation between the SIRS and the post-operative renal dysfunction; the cause is more likely an intra-operative insult, likely bypass related perfusion. Establishing this model allowed further research to be undertaken focusing on the attenuation of the injury described with reference to modification of the inflammatory response with omega-3 fatty acids.

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CHAPTER 4

A PRE-OPERATIVE INTRAVENOUS INFUSION OF OMEGAVEN ATTENUATES CARDIOPULMONARY INJURY IN THE FIRST 8 HOURS FOLLOWING PAEDIATRIC CARDIAC SURGERY

4.1 Introduction:

With advances in paediatric cardiac surgery, there has been an increase in complex corrective procedures, which are often performed in a younger, more vulnerable patient population. Although the mortality from paediatric cardiac surgery has decreased over the past two decades[1], significant morbidity from post-operative multiple organ dysfunction remains. The injury seen post-operatively was traditionally attributed to hypo-perfusion[2]; however, the role of the systemic inflammatory response in this injury is now widely appreciated[3] and is providing a target for possible organ protective strategies. Although much research has been undertaken, effective safe therapies have yet to translate from the laboratory into clinical practice.

Omega-3 fatty acids have been shown both to prevent ischemic injury and to attenuate the activation of the innate immune response which underlies SIRS, both of which are key events in cardiopulmonary bypass and circulatory arrest. Previous work in the laboratory in Beaumont Hospital by Dr Jonathan McGuinness demonstrated a 40% reduction in myocardial infarct size in a rabbit regional ischemia-reperfusion model pre-treated with an intravenous infusion of omega-3 fatty acids[4]. Cell work was used to elucidate the actions of the omega-3 fatty acid emulsion on the SIRS: a significant reduction in the inflammatory endothelial release of IL-8 and the expression of ICAM-1

was demonstrated following four hours of pre-treatment. The mechanism of this effect was shown to be due to a reduction in the inflammatory induced activity of NFkB, and an increase in protective HSP72 expression[5].

The aim of this study therefore was to determine if this observed protective effect translated to multi-organ protection in the setting of paediatric cardiac surgery within the first eight hours following reperfusion. The juvenile piglet cardiopulmonary bypass and deep hypothermic circulatory arrest model was established in the Biomedical Laboratory and was utilized for this purpose. Our specific objectives were:

- To determine if omega-3 fatty acids provided protection against the cardiac, pulmonary, renal, and cerebral injury following cardiopulmonary bypass and DHCA
- To determine if the mechanism of protection of omega-3 was due to a reduction in leucocyte-endothelial interactions, by measurement of white cell counts in peripheral blood and in the tissues on histology and immunohistochemistry.
- To provide a basis for a further more detailed study using the juvenile piglet bypass model extending the period of observation to 24 hours.

4.2 Materials and Methods:

The juvenile piglet model of cardiopulmonary bypass with ninety minutes of deep hypothermic circulatory arrest for this study, as described in detail in Chapter 2. Five animals received an infusion of 2mls/kg of Omegaven (Fresenius Kabi, Germany) over a four hour period immediately pre-operatively through a peripheral ear vein cannula.

Omegaven was chosen as the omega-3 fatty acid emulsion because it is clinically licensed for use in Europe; it is used as part of total parenteral nutrition. The dose is the daily dose recommended by the company. The infusion period of four hours prior to injury was found to be the optimal time for pre-treatment in previous cell work[2], and does not exceed the maximum rate of infusion recommended by the company. The heart rate, peripheral oxygen saturations and temperature of the piglets were monitored during the infusion period and maintained at physiological levels. Five animals received an infusion of 2mls/kg of 0.9% saline (Baxter, UK) over four hours to serve as controls.

Following the period of infusion, non-invasive and invasive monitoring of cardiac, pulmonary and renal function, and cerebral regional oxygen saturations was established. The heart was cannulated and cardiopulmonary bypass established. The piglets were cooled to 18°C and the circulation arrested for ninety minutes. Following this period, bypass was re-commenced, the piglets were warmed and then weaned from bypass, and the chest closed. The piglets were then observed under anaesthesia for eight hours. This time for observation was chosen as it was expected that the SIRS would become apparent within 4 – 6 hours and that differences between groups could be therefore be appreciated by 8 hours.

4.3 Results:

4.3.1 Organ Results:

Significant early organ dysfunction was observed in the heart, lungs and kidneys. At 8 hours, there was no appreciable cerebral injury on histology. Omega-3 protected against the cardiopulmonary injury, but did not have any effect on the renal injury in this study.

4.3.1.1 Cardiac Injury:

Cardiac injury was assessed using Millar cardiac catheter readings of left ventricular systolic and diastolic function at baseline (on opening of the chest) and two hourly thereafter; troponin levels at baseline and at three hours; organ wet:dry ratio; and histological examination.

Ventricular function:

Systolic function was increased from baseline readings throughout the period of observation. This is most likely secondary to the dopamine used when weaning the animals from bypass which was continued to three to four hours post operatively. There was no difference between the control and omega-3 groups. Early diastolic dysfunction was observed in our control group which was prevented with omega-3, as seen in the graphs below.

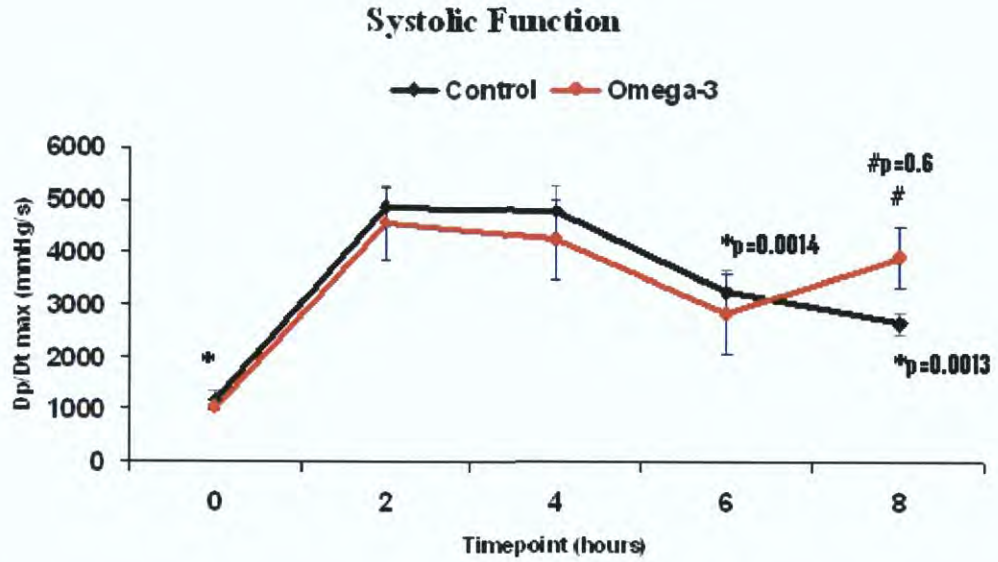

Figure 4.1.a

Systolic function was recorded at baseline prior to commencing cardiopulmonary bypass and then two hourly following reperfusion. Results are recorded as mean maximal change in pressure with respect to time (Dp/Dt max, mmHg/s) +/- SEM. Statistical analysis was with one way ANOVA and Bonferroni comparison of means. Systolic function was significantly elevated from baseline readings at all time points; there was no difference between the control and omega-3 groups.

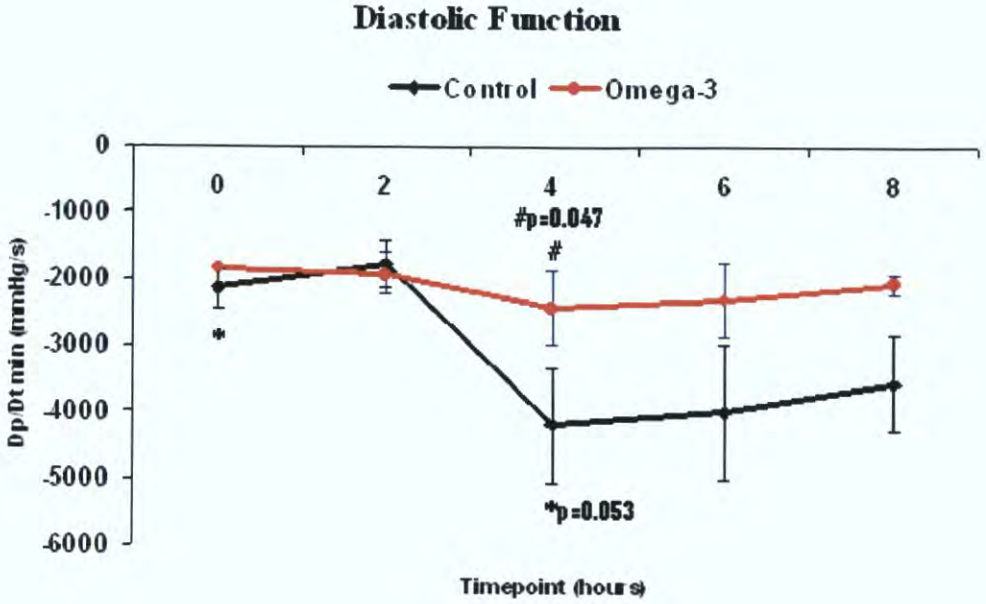

Figure 4.1.b

Diastolic function was also recorded at baseline and then every two hours following reperfusion. Results are reported as mean minimal change in pressure with respect to time (Dp/Dt min, mmHg) +/- SEM. Statistical analysis is with one way ANOVA and Bonferroni comparison of means. The diastolic function in the control animals trends to a reduction from baseline by four hours. In the omega-3 animals, this dysfunction is prevented.

Troponin:

Troponin I was measured on serum samples by the Biochemistry laboratory in Beaumont Hospital. There was no significant difference between the control and omega-3 groups.

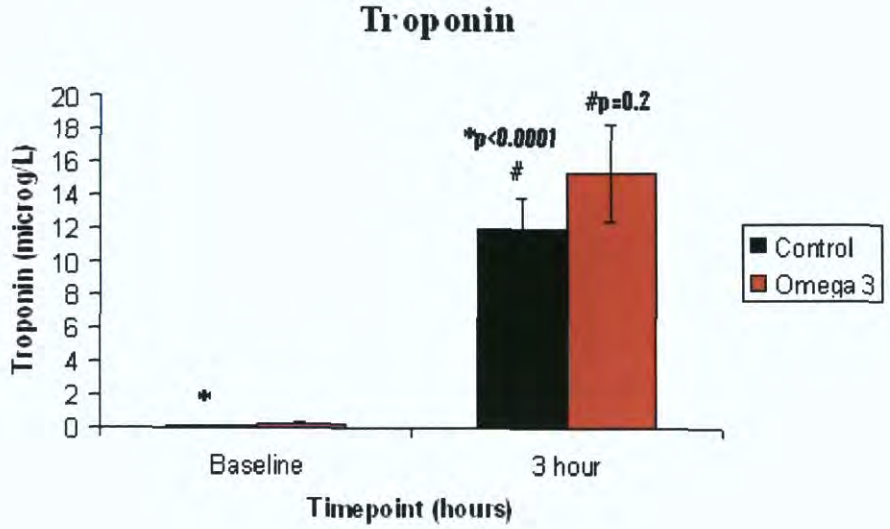

Figure 4.2

Troponin levels were measured at baseline and at 3 hours. Results are mean levels of troponin (µg/L) +/- SEM, statistical analysis was using a paired t test. While there was a significant increase in troponin levels from baseline to three hours, omega-3 pre-treatment did not attenuate this increase.

Wet: Dry ratio:

As measured by organ wet:dry ratio, there was no significant difference in cardiac oedema at eight hours between the control and omega-3 groups. Results are reported as mean ratio +/- SEM. Statistical analysis was with paired t test.

Control: 4.21 +/- 0.25 Omega-3: 4.61 +/- 0.09

P = 0.1 (paired t test)

Histology:

There was no significant difference in the histological appearance of the control and omega-3 hearts. The histological features examined are detailed in Chapters 2 and 3, but include cellular degeneration and necrosis, oedema and inflammatory cell infiltrate. A composite histological injury score was obtained and compared between the groups. These results and representative images of the myocardium are shown below.

Composite injury score:

Results are reported as mean +/- SEM. Statistical analysis was with the paired t test.

Control: 7 +/- 1.1 Omega-3: 6.2 +/- 1.2

P=0.298 (paired t test)

Representative Images:

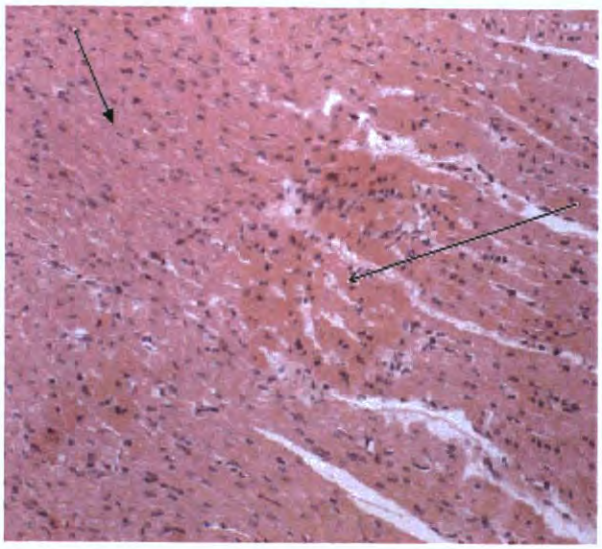

Figure 4.3.a
Mycocardium. The short arrow indicates normal myocytes; the long arrow indicates degenerate myocytes.

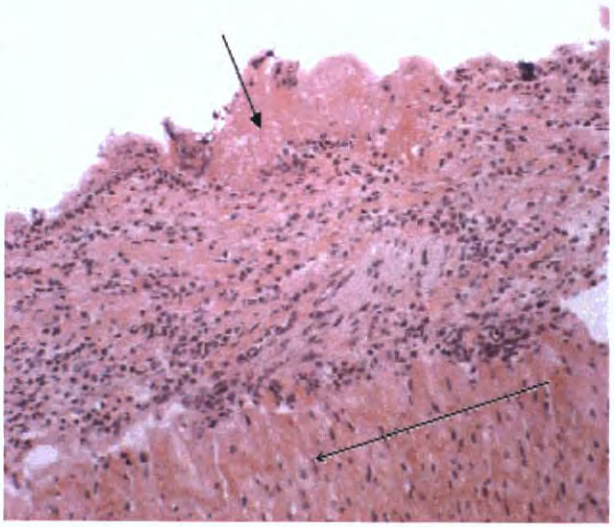

Figure 4.3.b
This shows myocardium (long arrow) with surface fibrin (short arrow) and inflammatory cells.

4.3.1.2 Pulmonary Injury:

Pulmonary injury was assessed by measurement of dynamic and static compliance, using the CO_2SMO Plus respiratory profile system attached to the endotracheal tube, at baseline and hourly following reperfusion; partial pressure of oxygen (pO_2) in arterial blood gas samples two hourly; organ wet:dry ratio; and histological examination.

Pulmonary compliance:

Pulmonary compliance is a measure of the ability of the lung to expand under pressure, as measured by volume change per unit of pressure change (mls/cmH_2O). As this measurement is affected by lung volumes, tidal volumes were maintained at standardized levels of 150mls for animals less than 15kgs, and 10mls/kg for animals over this weight.

All study animals demonstrated a reduction in compliance post-operatively. However at eight hours, a statistically significant improvement was observed in animals pre-treated with omega-3 as shown in the graphs below. At this early stage, this improvement in compliance did not translate to an improvement in oxygenation as measured by partial pressure of oxygen.

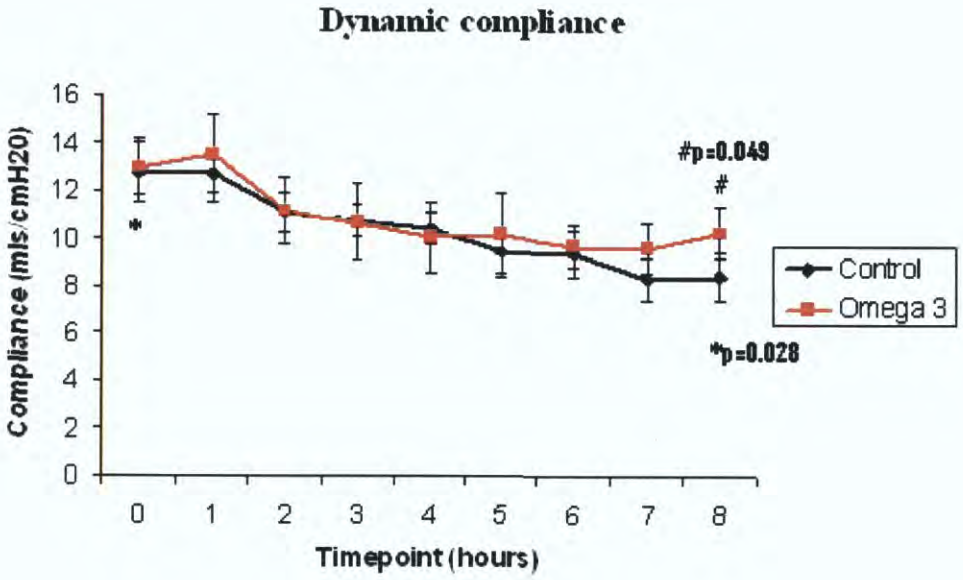

Figure 4.4

Dynamic compliance was recorded at baseline and hourly thereafter. Results are reported as mean compliance (mls/cmH_2O) +/- SEM; statistical analysis comparing change from baseline to eight hours was with one way ANOVA and Turkey comparison of means; statistical analysis comparing the control and omega-3 groups at eight hours was with a paired t test. Over the eight hour observation period, compliance in the control group decreased significantly from baseline (baseline:

12.74 +/- 1.24 mls/cmH_2O; 8 hours: 8.38+/-1.06 mls/cmH_2O; p=0.028, ANOVA, Turkey). However from four to six hours, the compliance of the omega-3 animals is seen to stabilize, with an improvement observed at eight hours. Compliance was significantly better in the omega-3 group compared to controls at this time-point (controls: 8.38 +/- 1.06 mls/cmH_2O; omega-3: 10.22 +/- 1.04 mls/cmH_2O; p = 0.049, paired t test).

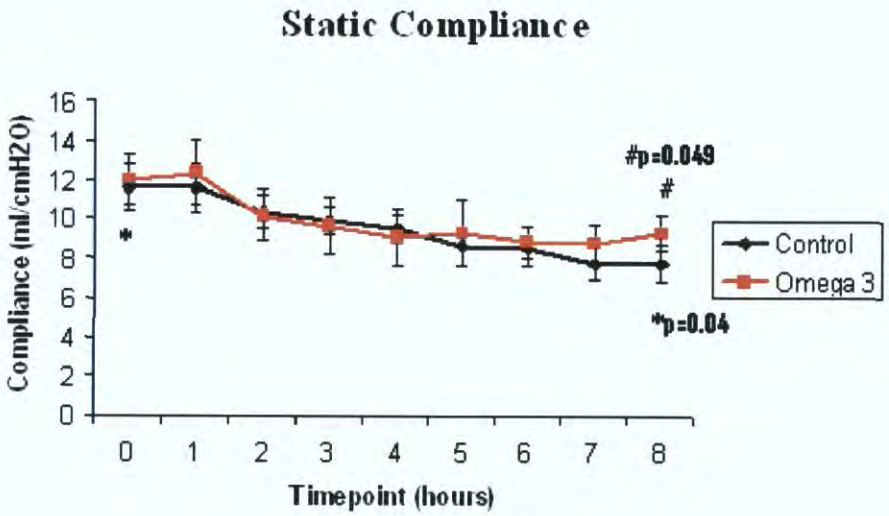

Figure 4.5

Static compliance was recorded at baseline and hourly thereafter. Results are reported as mean compliance (mls/cmH_2O) +/- SEM; statistical analysis is with one way ANOVA and Turkey comparison of means for change from baseline to eight hour readings, and with paired t test for comparison of the control and omega-3 groups at eight hours. A similar pattern is seen with static compliance, with a reduction in the compliance of the controls from baseline to eight hours (baseline:

11.62 +/- 1.18 mls/cmH_2O; 8 hours: 7.78 +/- 0.96 mls/cmH_2O; p=0.04, ANOVA, Turkey). Again at 8 hours the improved static compliance in the omega-3 group is statistically significant compared to controls (controls: 7.78 +/- 0.96 mls/cmH_2O; omega-3: 9.38 +/- 0.93 mls/cmH_2O; p = 0.04, paired t test).

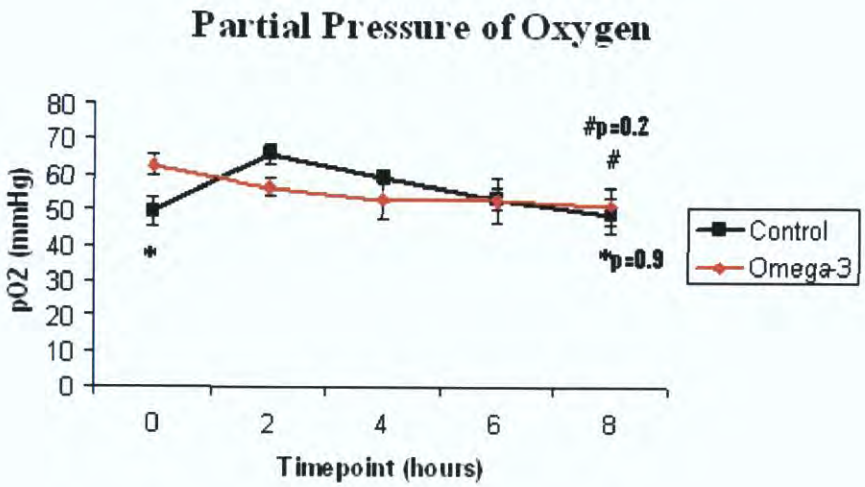

Figure 4.6

PO_2 was measured two hourly on arterial blood gas analysis. Results are reported as mean partial pressure of oxygen (mmHg) +/- SEM; statistical analysis comparing change from baseline to 8 hour values is with one way ANOVA and Turkey comparison of means; statistical analysis between the control and omega-3 groups at 8 hours is with the paired t test. There was no difference in pO2 from baseline to 8 hours in the controls (p=0.9, ANOVA, Turkey); nor was there any difference between the omega-3 and control groups at 8 hours (p=0.2, paired t test).

Wet: Dry Ratio:

There was no significant difference in pulmonary oedema at eight hours as measured by organ wet:dry ratio. Results are reported as mean ratio +/- SEM. Statistical analysis was with the paired t test.

Control: 6.39 +/-1.49 Omega-3: 6.7 +/- 0.64

P = 0.4 (paired t test)

Histology:

There was no significant difference in the histological features of the lungs pretreated with omega-3. The composite injury scores and representative images of lung histology demonstrating significant neutrophil infiltration are shown below.

Composite injury score:

Results are reported as mean +/- SEM. Statistical analysis was with the paired t test.

Control: 10.6 +/- 0.93 Omega-3: 12.6 +/- 1.96

P=0.23 (paired t test)

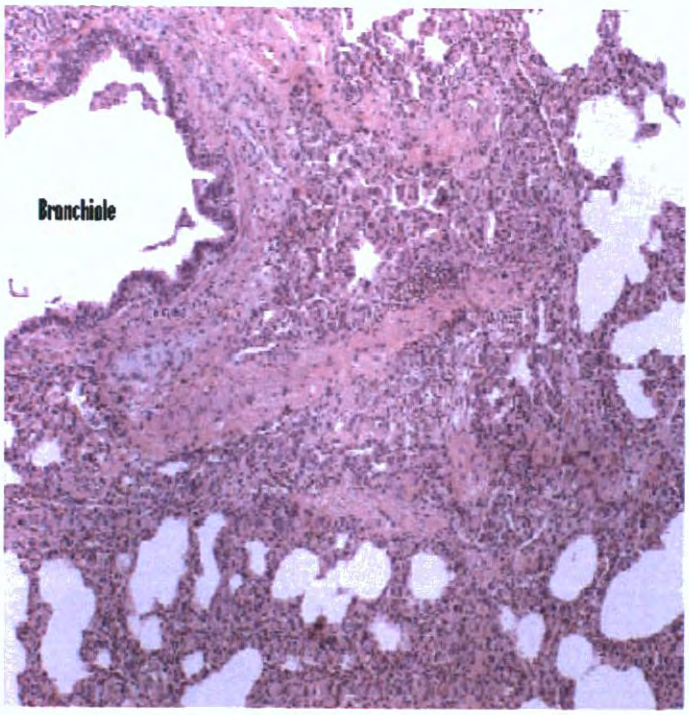

Figure 4.6 a
lung histology showing thickening of the interstitium (space between alveoli), reduced size of alveolar spaces, and inflammatory cell infiltrate.

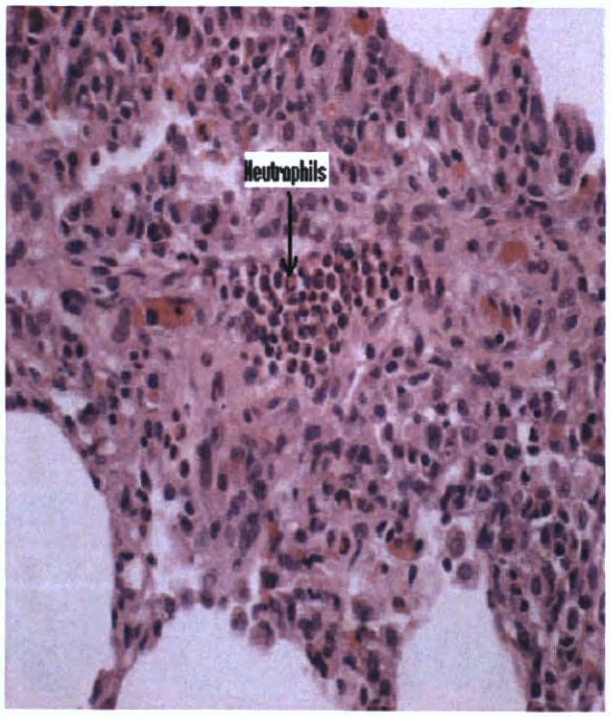

Figure 4.6.b
Higher power view showing the inflammatory cells.

4.3.1.3 Renal Injury:

Renal injury was assessed with hourly urine output measurements, urinary N-acetyl glucosaminadase (NAG), fractional excretion of urinary sodium, organ wet:dry ratio, and histological examination. There was no significant protective effect in the observed organ injury with omega-3 pretreatment as shown on the graphs below.

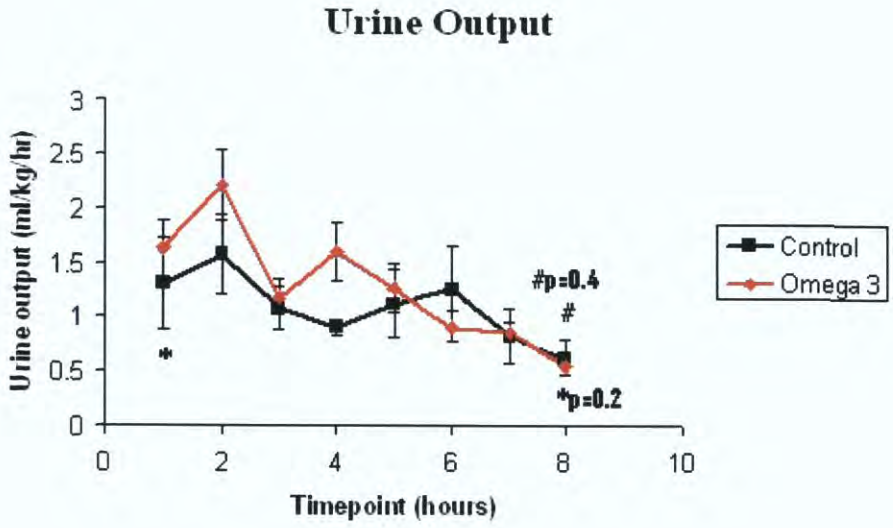

Figure 4.7

Urine output was measured hourly from 1 hour post re-commencement of cardiopulmonary bypass. Results are reported as mean urine output (ml/kg/hr) +/- SEM; statistical analysis is with one way ANOVA and Turkey comparison of means for detecting changes from baseline to 8 hours, and with paired t test for comparison of the control and omega-3 groups at 8 hours. Although a reduction in urine output was observed as can be seen on the graph, the decrease from baseline to 8 hours was not statistically significant (p = 0.2, ANOVA, Turkey). There was no difference between the control and omega-3 groups.

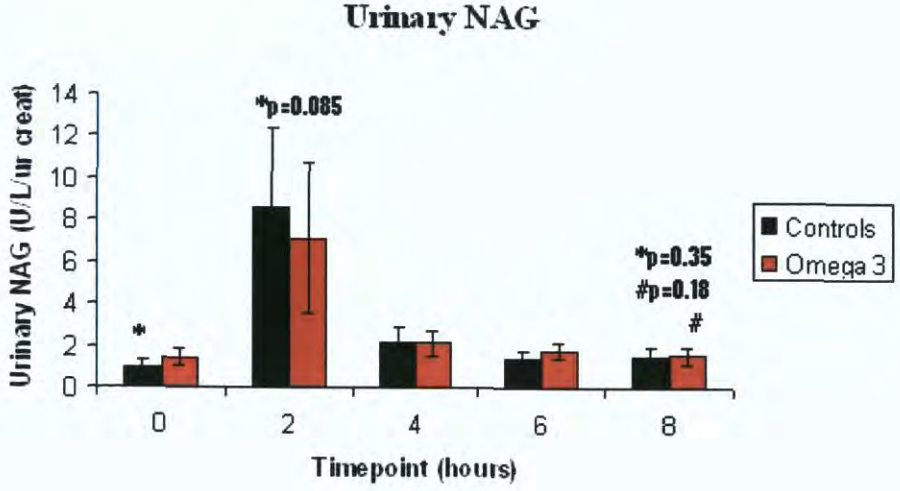

Figure 4.8

Urinary NAG was measured at baseline and 2 hourly thereafter. Results are reported as mean volume activity of urinary NAG (U/L) indexed to urinary creatinine (mg/L) +/- SEM. Statistical analysis is with one way ANOVA and Turkey comparison of means for detecting changes in controls from baseline to 2 hours and to 8 hours; for comparison of differences between the omega-3 and control groups at individual time points, a paired t test was used. Despite a trend towards an increase in urinary NAG at two hours in both groups, this rise was not statistically significant (p=0.085, ANOVA, Turkey). There were no significant differences between the groups at any time point.

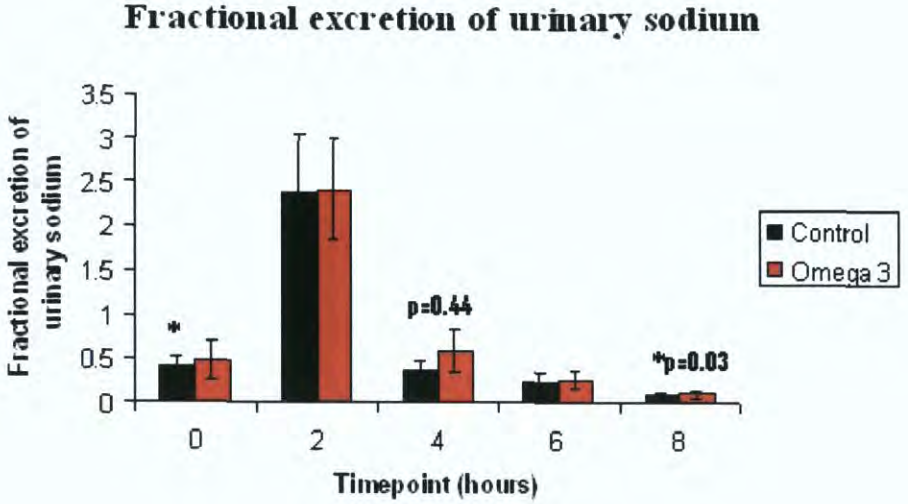

Figure 4.9

Fractional excretion of urinary sodium was measured at baseline and then two hourly thereafter using the formula: {(urinary sodium/serum sodium) x (serum creatinine/urinary creatinine)} x 100. Results are reported as mean fraction +/- SEM; statistical analysis for comparison of groups at each time point was with the paired t test; statistical analysis from baseline to eight hours in the control group was with one way ANOVA and Turkey comparison of means. Fractional excretion of urinary sodium was elevated above 1 at two hours (indicating loss of the reabsorption capacity of the tubules). There was no significant difference in fractional excretion of urinary sodium between the control and omega-3 groups.

Wet: dry ratio:

As measured by organ wet:dry ratio, there was no significant difference in renal oedema between the two groups. Results are reported as mean ratio +/- SEM. Statistical analysis was with the paired t test.

Control: 5.78 +/- 0.17 Omega-3: 5.64 +/- 0.14

P = 0.2 (paired t test)

Histology:

There was no significant difference in histological features of the control and omega-3 pretreated renal samples. The composite histological injury score and a representative image of the kidney at eight hours are shown below.

Composite injury score:

Results are reported as mean +/- SEM. Statistical analysis was with the paired t test.

Control: 5 +/- 1.22 Omega-3: 5.4 +/- 1.44

P=0.42 (paired t test)

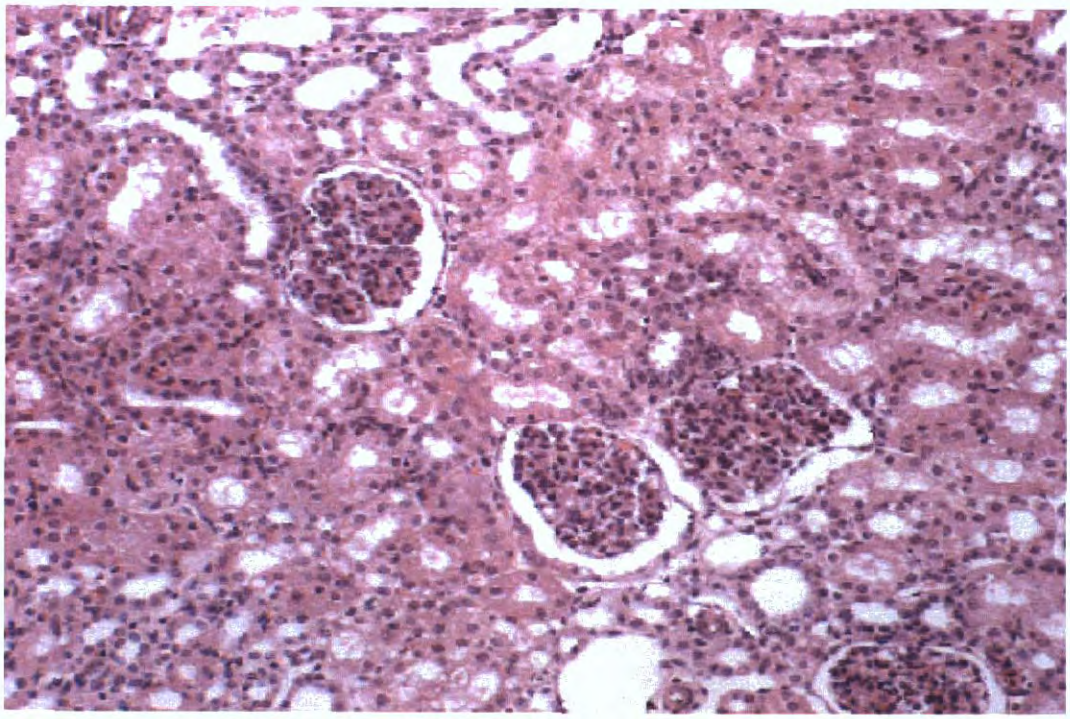

Figure 4.10
Kidney showing glomeruli and tubules. The glomeruli are more cellular than expected as they contain some inflammatory cells.

4.3.2 No difference in perfusion between the two groups:

Both groups demonstrated stable hemodynamics throughout the eight hour observation period, with no significant differences in heart rate, mean arterial blood pressure and central venous pressure as shown on the following graphs. Inotropic and fluid bolus requirements were similar between the groups, with no statistically significant differences.

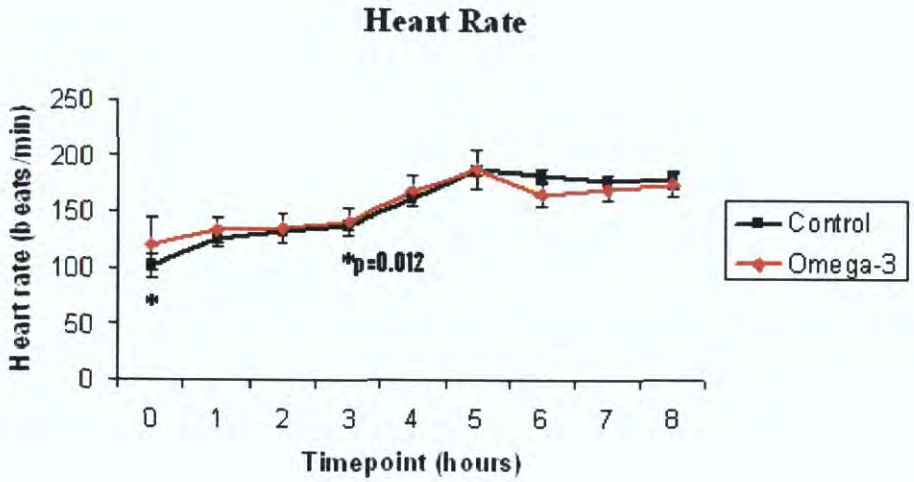

Figure 4.11

Heart rate was recorded hourly from the femoral arterial line reading. Results are reported as mean heart rate (beats/min) +/- SEM. Statistical analysis was with one way ANOVA and Turkey comparison of means for changes from baseline to eight hours; paired t tests were used to compare omega-3 and control groups. There were no differences between the control and omega-3 groups. However, from 3 hours on, a statistically significant tachycardia developed in both groups which was sustained throughout the period of observation.

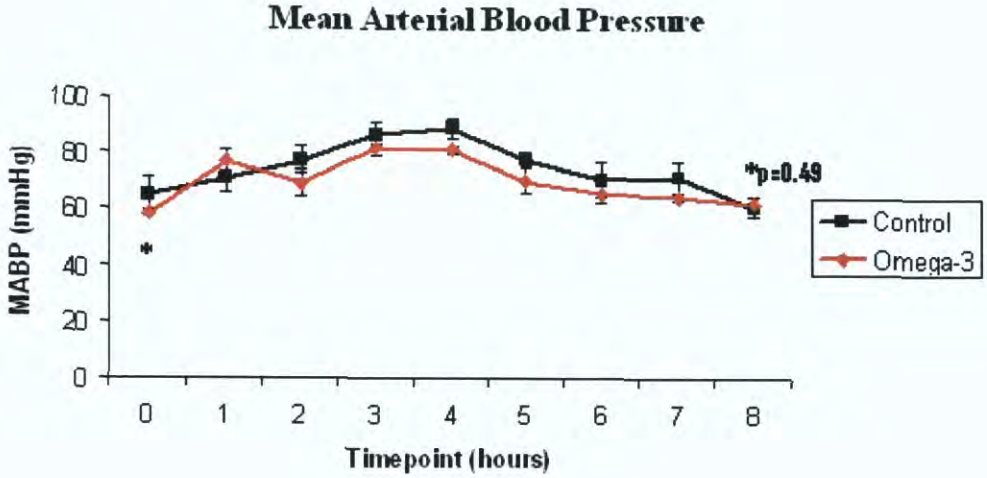

Figure 4.12

Mean arterial blood pressure was recorded hourly from the femoral arterial line. Results are reported as mean MABP (mmHg) +/- SEM; statistical analysis was with one way ANOVA and Turkey comparison of means for comparisons along the time line, and with paired t tests for comparisons between the groups. As can be seen from the graph, mean arterial blood pressure did not drop below the critical value of 60mmHg at any time. There was no significant difference in the control group from baseline to 8 hours (p=0.49, ANOVA, Turkey); nor were there any differences between the control and omega-3 groups.

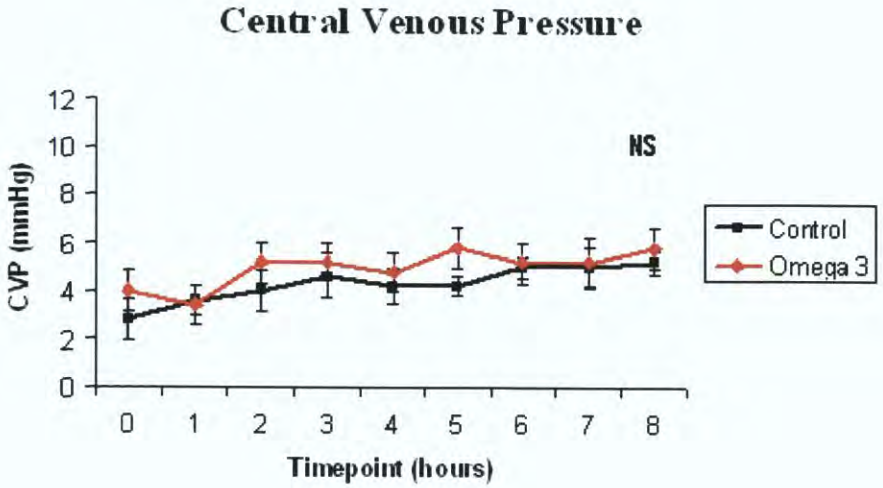

Figure 4.13

Central venous pressure was recorded hourly from the central venous line. Results are reported as mean central venous pressure (mm Hg) +/- SEM. Statistical analysis was with paired t tests to compare groups. All animals had an adequate CVP at all times, with no differences between the groups.

Lactate and base excess were measured as markers of anaerobic metabolism in the tissues. Again there were no significant differences between the groups.

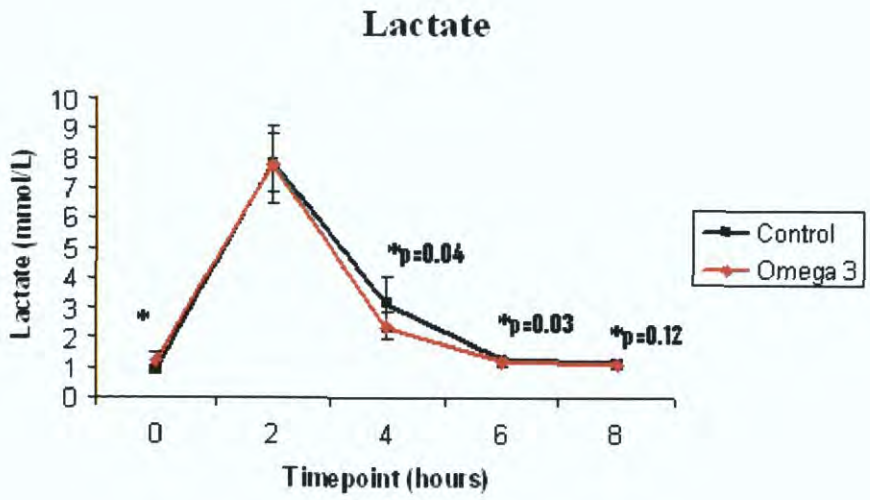

Figure 4.14

Lactate was measured two hourly on arterial blood gas. Results are reported as mean lactate (mmol/L) +/- SEM. Statistical analysis was with ANOVA, Turkey comparison of means and paired t tests. An initial washout phenomenon was observed immediately post bypass, with levels returning to baseline by 6-8 hours. There were *no significant* differences between the groups at any time point.

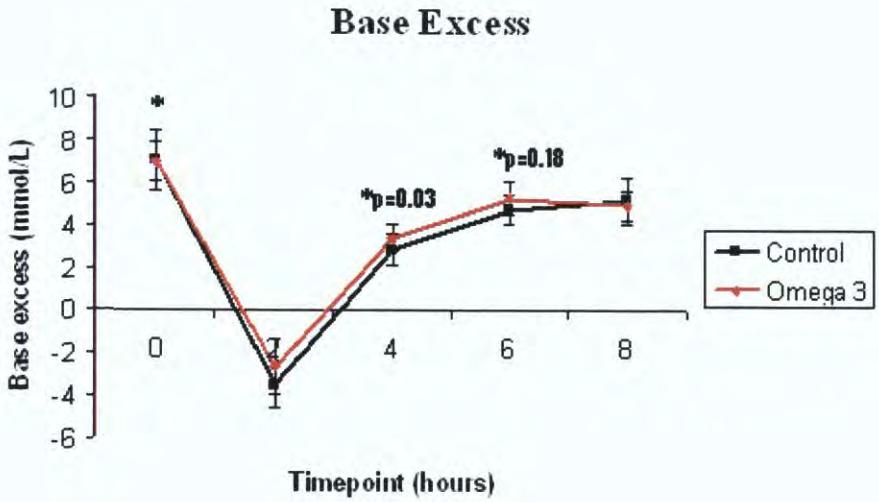

Figure 4.15

Base excess was measured two hourly on arterial blood gas. Results are reported as mean base excess (mmol/L) +/- SEM. Statistical analysis was with ANOVA and Turkey comparison of means, and with paired t tests. A similar pattern to the lactate result was seen: an initial washout phenomenon post bypass with subsequent return to baseline levels by 4 – 6 hours. There was no difference between the control and omega-3 groups.

Tissue oxygenation was assessed with mixed venous oxygen saturations (from central venous blood gas measurements), and renal and cerebral regional oxygen saturations (NIRS). Again there was no significant difference between control and omega-3 animals.

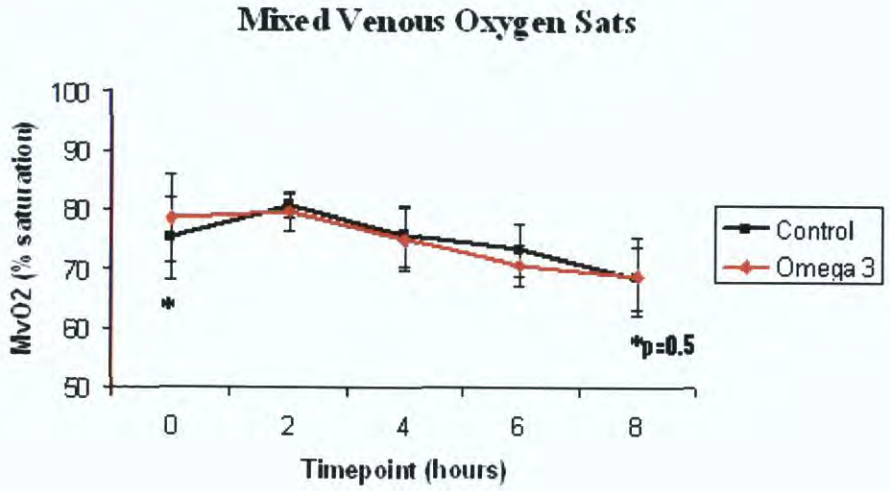

Figure 4.16

Mixed venous oxygen saturations were recorded two hourly from arterial blood gas analysis. Results are reported as mean mixed venous oxygen saturation (percentage) +/- SEM. Statistical analysis was with one way ANOVA and Turkey comparison of means for changes from baseline to 8 hours in the control group, and with paired t test for comparison between the groups at each time point. Mixed venous oxygen saturations were stable throughout the observation period in all animals remaining above the critical level of 65%, and there was no observed difference between the control and omega-3 groups.

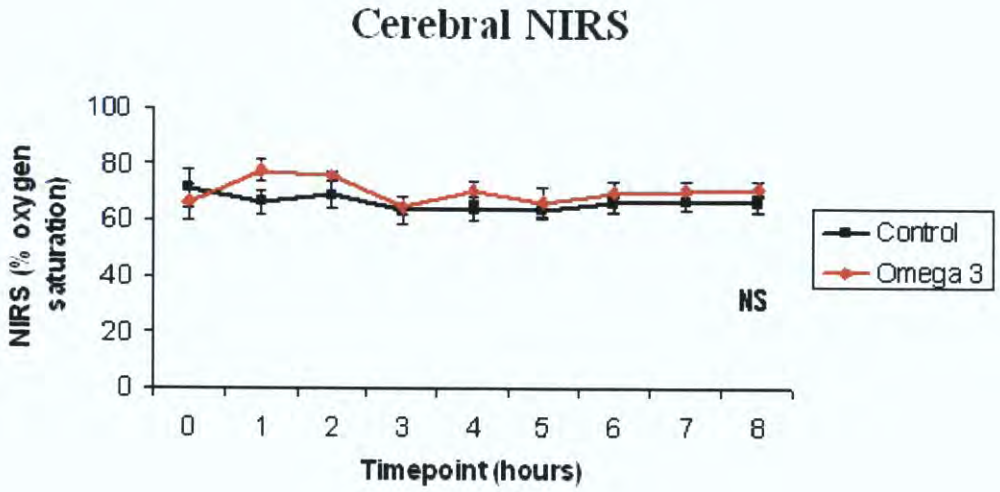

Figure 4.17

Cerebral near infrared spectroscopy (NIRS) readings were recorded hourly. Results are reported as mean percentage regional oxygen saturation +/- SEM; statistical analysis is with one way ANOVA and Turkey comparison of means for changes from baseline to 8 hours, and with the paired t test for comparison of the control and omega-3 groups at 8 hours. There was no significant difference in cerebral NIRS readings over the 8 hour observation period (p=0.5, ANOVA, Turkey), and there was no difference between the two groups at 8 hours (p=0.1, paired t test).

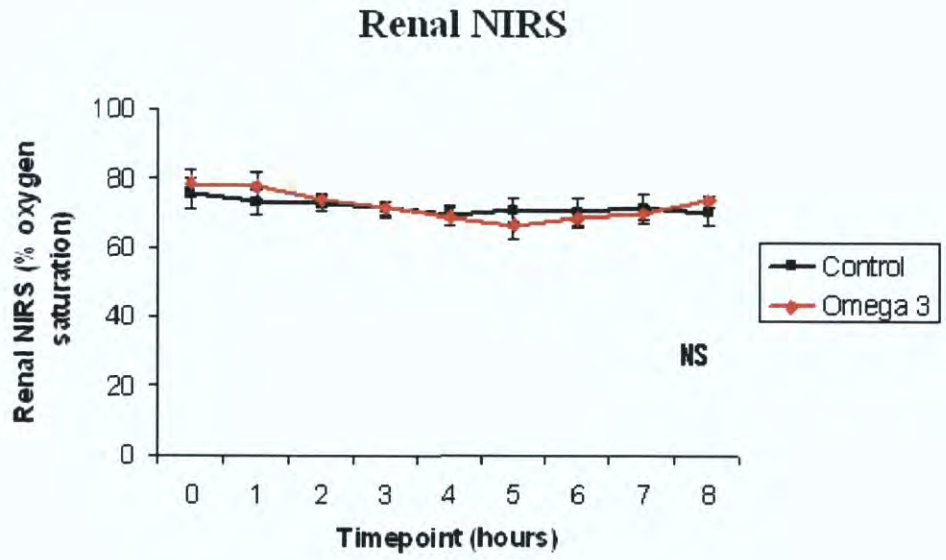

Figure 4.18

Renal near infrared spectroscopy (NIRS) readings were recorded hourly. Results are reported as mean percentage regional oxygen saturations +/- SEM; statistical analysis is with one way ANOVA and Turkey comparison of means for changes from baseline to 8 hours, and with the paired t test for comparison of control and omega-3 groups at 8 hours. Again, the renal regional oxygen saturations were stable throughout the observation period in all animals with no significant differences between the groups.

4.3.3 Markers of inflammation

White cell count measurements:

Blood samples were taken at baseline, and then at 15 minutes, 30 minutes, 1 hour and 6 hours following the re-institution of cardiopulmonary bypass. The results are shown on

the graph below. The baseline, 15 minute, 30 minute and 1 hour readings showed no significant difference between the two groups; however at 6 hours, the omega-3 animals had a significant reduction in peripheral white cell count.

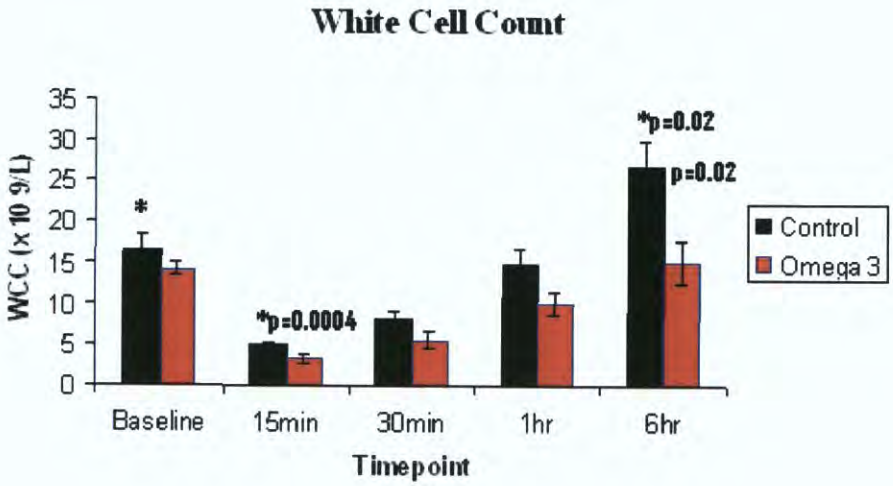

Figure 4.19

Peripheral white cell count was measured at baseline, and then at 15 mins, 30 mins, 1 hour and 6 hours post re-commencement of bypass. Results are reported as mean white cell count (white cells x 10^9/L) +/- SEM; statistical analysis was with one way ANOVA and Turkey comparison of means for changes in the control group from baseline to 15 minutes and 6 hours, and with the paired t test for comparison between the groups at 6 hours. In the control group, an initial drop in WCC from baseline to 15 minutes is seen (p=0.0004, ANOVA, Turkey) with a subsequent rise at 6 hours (p=0.02, ANOVA, Turkey). The initial reduction is similar in the omega-3

group, however at 6 hours the increase in WCC is significantly less in the omega-3 group compared to controls (p=0.02, paired t test).

Myeloperoxidase levels:

Myeloperoxidase, a tissue damaging enzyme released by activated neutrophils, was measured by immunohistochemistry in the harvested organs of control and omega-3 animals. Animals in the omega-3 group had significantly lower levels of myeloperoxidase as shown on the graph below.

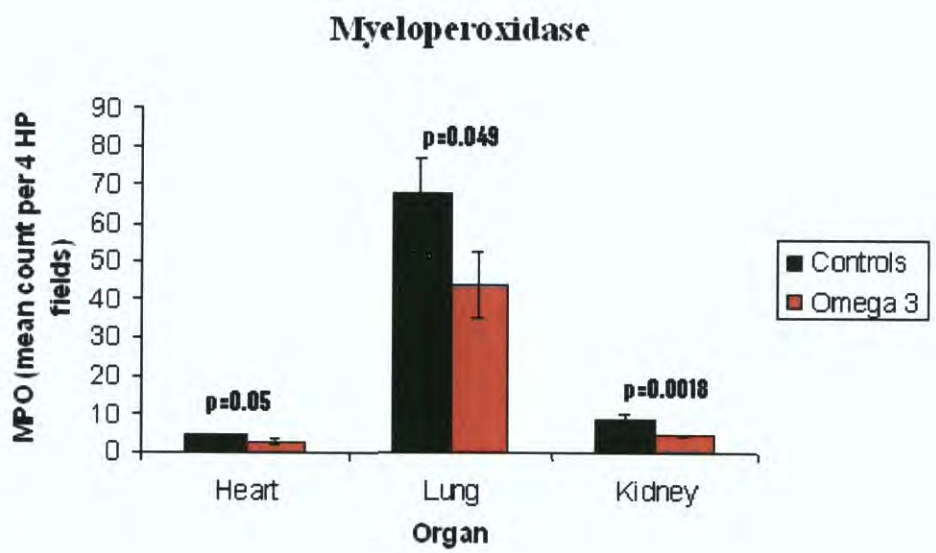

Figure 4.20

Myeloperoxidase was measured in each organ using immunohistochemisty. Results are reported as mean count over 4 high power fields +/- SEM. Statistical analysis

was with paired t tests. A significant reduction in the MPO level in each organ is observed with omega-3 pre-treatment.

Representative images comparing control and omega-3 lung samples stained for myeloperoxidase are shown below.

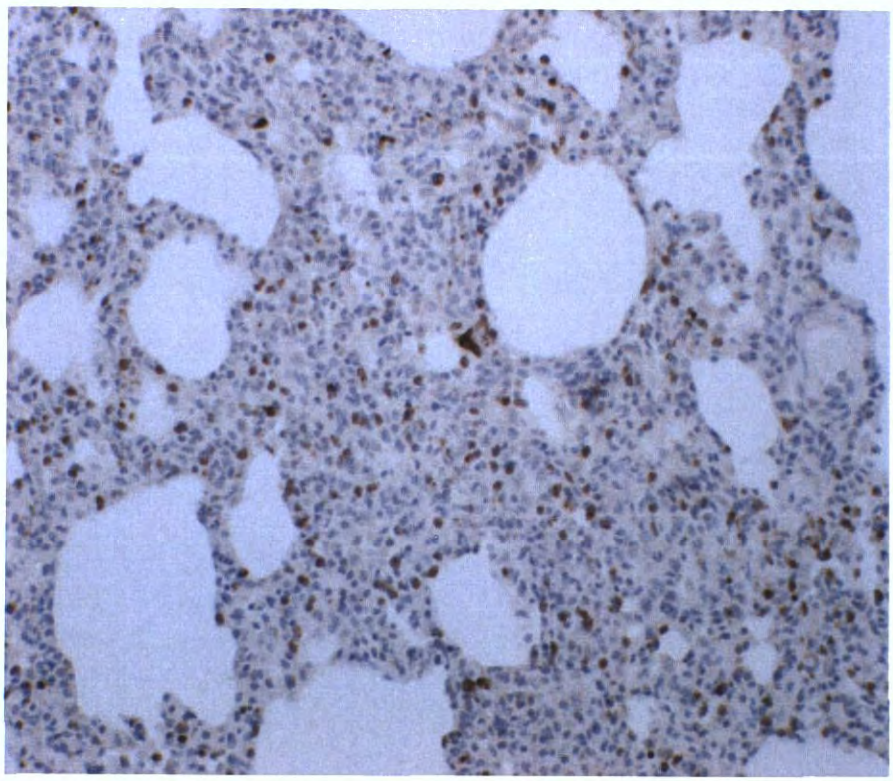

Image 1: Control lung sample showing alveolar septal thickening and predominant staining for MPO (brown stains)

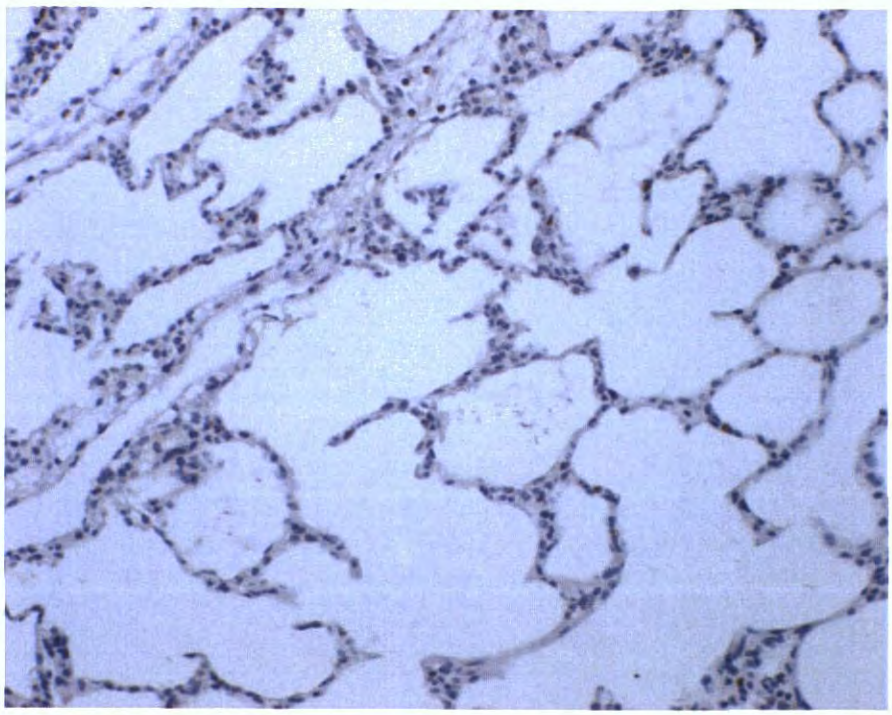

Image 2: Omega-3 lung sample demonstrating much less MPO staining

4.4 Discussion:

With the advances in paediatric cardiac surgery, increasingly complex procedures are being performed in a younger, more vulnerable patient population. Reduced mortality rates over the last number of years have led us to focus now on reducing the morbidity in these patients. The understanding of the SIRS as a major factor in the pathology of post-operative multiple organ dysfunction has provided a new target for organ protection. Studies using omega-3 fatty acids in a number of settings have demonstrated anti-inflammatory and anti-infarct effects. Previous in vitro and in vivo work in our laboratory

demonstrated a reduction in the SIRS pathophysiology and a reduction in myocardial infarct size following pre-treatment with omega-3 fatty acids[4,5]. The aim of this study was to determine if these effects translated into multiple organ protection in a model of paediatric cardiac surgery. To this end, the juvenile piglet model of cardiopulmonary bypass and circulatory arrest was employed and a single four hour infusion of omega-3 fatty acids administered pre-operatively.

With regard to cardiac function, a trend towards impaired diastolic function was observed in the early post-operative period in control animals in this study. There was no impairment of systolic function, which was in fact improved from baseline readings over the period of observation. Both animal and clinical studies have repeatedly demonstrated transient early post-operative ventricular dysfunction. Clinical studies following coronary artery bypass grafting demonstrate that 90 – 96% of patients have a reduction in both right and left ventricular ejection fraction, maximally between 2 and 6 hours post-operatively[6,7]. In a study using the juvenile piglet bypass model, systolic dysfunction peaked at 4 hours of reperfusion, returning to normal at 6 hours; diastolic dysfunction peaked slightly later at 6 hours[8]. Therefore, it would have been expected to see both systolic and diastolic dysfunction in the control animals over the 8 hour period of observation. However, two factors may have accounted for the stable ventricular function in this study. Firstly, there was no operative intervention on the heart. Secondly, all the study animals were weaned from bypass with a continuous infusion of dopamine which was continued until an average of three hours. A clinical study of CABG patients found that post-operative therapy with inotropes delayed but did not prevent the occurrence of

post-operative ventricular dysfunction[4]. Therefore, it is possible that the study time period was too short to observe systolic dysfunction. In the omega-3 pre-treated animals, diastolic function was preserved. Improved diastolic function has been demonstrated on echocardiography in a clinical study of 224 men receiving oral supplementation with EPA and DHA for seven weeks[9]. In addition, animal studies using isolated rat hearts following 12 - 16 weeks of oral supplementation with omega-3 fatty acids demonstrated increased contractile recovery following ischemia and reperfusion[10,11]. This effect is most likely due to modification of the fatty acid composition of phospholipids in cell membranes. However, prevention of diastolic dysfunction may also be due to the attenuation of SIRS with Omega-3.

With regard to pulmonary function, improved compliance at eight hours was demonstrated in animals pretreated with omega-3 fatty acids. Both animal and clinical studies have demonstrated the benefits of omega-3 fatty acids on pulmonary indices in various settings. A recent study in fat-1 mice (who endogenously convert omega-6 to omega-3 fatty acids) showed improved lung compliance in a model of acute lung injury[12]. In ventilated patients with acute lung injury, enteral feed supplemented with omega-3 fatty acids results in improved pulmonary compliance[13] and oxygenation[14]. Similarly, improved pulmonary status was seen in patients with severe sepsis/septic shock feed an enteral diet supplemented with omega-3 fatty acids[15]. At this early stage in this study, the improved compliance noted did not translate into improved oxygenation. This was one of the reasons the decision was taken to extend the study observation period to 24 hours - to

see if the observed improvement in compliance was sustained, and if it subsequently resulted in improved oxygenation indices.

Renal injury was demonstrated in a trend towards a reduced urine output over the observation period, and an early increase, at two hours post reperfusion, in urinary NAG and fractional excretion of urinary sodium. This pattern of early increase in these two markers, which are indicative of tubular injury, has been described in previous studies[16]. As this injury occurred early, we hypothesized that this was not due to SIRS, as this would not be expected to be maximally present until 4 – 6 hours post-operatively. Our omega-3 animals did not show any improvement in the measured renal parameters which we might expect from the different mechanism involved. Previous studies with omega-3 fatty acids have demonstrated beneficial effects on renal function: however, this has usually been at a later time point. A recent study in mice orally supplemented with omega-3 fatty acids and then subjected to renal ischemia-reperfusion demonstrated normal serum creatinine levels at 24 hours post injury in contrast to control animals fed on a normal diet who showed a significant rise in serum creatinine[17]. A clinical study of patients with severe pancreatitis parenterally supplemented with omega-3 fatty acids showed a reduction in the number days of renal replacement therapy necessary; this was following six days of supplementation[18]. It is possible therefore that any protective effect of omega-3 fatty acids on renal function was yet to occur in our study, which again provided a basis for a second study with a longer period of observation.

At all times during the study, perfusion was adequate in all animals as demonstrated by stable mean arterial blood pressure and central venous pressure. As there was no evidence of volume depletion, the tachycardia seen from 3 hours onwards was likely a direct manifestation of the SIRS. Lactate and base excess, measures of anaerobic metabolism, showed an initial washout phenomenon, but levels had returned to baseline by 4 - 6 hours and remained stable throughout the remainder of the observation period. Mixed venous oxygen saturations, a measure of oxygen uptake by the tissues, was stable in both groups at all times. Both cerebral and renal NIRS readings were also stable throughout, indicative of good perfusion of the brain and kidney at all times. Thus, hypo-perfusion was not the cause of the injury seen in this study.

White cell count measurements demonstrated an initial drop at fifteen minutes, with levels returning to normal at one hour post reperfusion. This could be indicative of initial sequestration in the tissues: this pattern has been elucidated previously in a clinical study which measured myocardial and peripheral white cell counts[19], or could be just a simple manifestation of haemodilution with bypass. A subsequent rise in white cells is secondary to release of immature granulocytes from the bone marrow[5]. This rise was significantly attenuated in the omega-3 animals, indicative of a reduction in the inflammatory response at this point. This finding is supported by the observed significant reduction in immunohistochemical staining for myeloperoxidase, a tissue damaging enzyme released by activated neutrophils in the tissues, in the omega-3 animals.

To summarize therefore, this study observing the piglets to 8 hours post reperfusion demonstrated significant early injury in the cardiac, pulmonary and renal systems. Omega-3 protected against diastolic dysfunction at four hours and led to improved pulmonary compliance at eight hours. This appeared to be related to a reduction in the severity of SIRS, as evidenced by a reduction in WCC rise and reduced levels of MPO stained cells in the lungs. The hypothesis at this point was that much of the expected injury was yet to become apparent – for example, pulmonary compliance only became statistically significantly reduced from baseline at eight hours, and partial pressure of oxygen was still stable at this time; also a low cardiac output syndrome had not yet developed in any of our animals. It was expected therefore that with a longer period of observation post-operatively, further injury would be seen in our control animals, thus allowing for further beneficial effects of the omega-3 infusion to become apparent. It was also hypothesized whether a second dose of omega-3 fatty acids 24 hours prior to surgery would enhance the benefits seen thus far and perhaps confer additional benefits in terms of renal injury or our other cardiac or pulmonary markers. This was based on the observation that the previous work carried out in our laboratory using the rabbit regional ischemia model which showed a 40% reduction in myocardial infarct size had a four day pre-operative omega-3 infusion regime[1]. Therefore, a protocol for the juvenile piglet cardiopulmonary bypass and DHCA model which incorporated a 24 hour period of observation following reperfusion post bypass was developed. A more detailed assessment of organ function, and also measurements of the SIRS and tissue effects of omega-3 fatty acids was incorporated in order to determine the underlying mechanism of the observed protection.

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CHAPTER 5

TWO PRE-OPERATIVE INTRAVENOUS INFUSIONS OF OMEGAVEN ATTENUATES THE SIRS IN THE FIRST 24 HOURS FOLLOWING PAEDIATRIC CARDIAC SURGERY

5.1 Introduction:

Having demonstrated an improvement in cardiopulmonary dysfunction at eight hours with a single 4 hour pre-operative infusion of omega-3 fatty acids, the hypothesis was that this improvement could be further increased if a second infusion was added 24 hours earlier. This was based on the previous work of Dr McGuinness in this laboratory in which 4 days of intravenous omega-3 produced a 40% reduction in myocardial infarct size in a rabbit regional ischemia-reperfusion model[1]. In addition, we extended the period of observation to 24 hours in order see if the beneficial effects noted were sustained. The injury observed in the control animals over this time period has been described in detail in Chapter 3. In particular in this study, the aim was to examine if the observed improvement in pulmonary compliance at eight hours continued and if it translated into improved oxygenation over the following hours, and if the low cardiac output syndrome observed could be attenuated. With the complex mechanisms of renal injury, although an improvement in the measured renal indices was not seen in the eight hour study, it was hoped that a later improvement may be observed.

The anti-inflammatory benefits of omega-3 are multiple. Studies have demonstrated reductions in pro-inflammatory cytokines and an increase in anti-inflammatory cytokines[9-13]; and a reduction in the activation of NFkB[2,3]. The mechanisms of this protection are thought to be as a result of the incorporation of the omega-3 fatty acids into the cell membranes, resulting in an increase in the omega-3/omega-6 fatty acid ratio[16,17]. Then, on inflammatory stimulation, there is an increase in the production of the anti-inflammatory eicosanoids derived from the omega-3 fatty acids (prostanoids of the 3 series and leukotrienes of the 5 series), and a concomitant reduction in the production of the pro-inflammatory eicosanoids derived from arachidonic acid (prostanoids of the 2 series and leukotrienes of the 4 series)[14,15]. Another protective mechanism of omega-3 fatty acids is through the reduction in the period of immunosuppression seen following cardiac surgery[24,25]. This period occurs following the acute phase of inflammation and is a result of the natural induction of anti-inflammatory cytokines. It results in a reduction in cellular immunity, with a shift in the balance between T helper cells towards the anti-inflammatory TH2 cells[21,22]. Omega-3 fatty acids have been shown to attenuate this period of immunosuppression. In addition, pretreatment with omega-3 fatty acids induces preconditioning as shown in previous cell work in our laboratory through the induction of HSP72 and NFkB inhibition with subsequent reduction in the cytokines IL-6 and IL-8, and cell adhesion molecule expression[10].

The specific objectives of this study therefore were as follows:

- To determine if the observed cardiopulmonary protection in the 8 hour study with omega-3 translated into further beneficial effects over an extended period of observation
- To determine if the addition of a second infusion 24 hours pre-operatively increased these benefits
- To determine if any protective effects of omega-3 on renal function became apparent
- To examine the effects of omega-3 fatty acids on the SIRS through the measurement of white cell counts and the cytokines IL-6, IL-8 and IL-10
- To examine the effects of omega-3 on eicosanoid production by measuring Leukotriene B_4
- To examine the effects of omega-3 on NFkB

5.2 Materials and Methods:

The juvenile piglet bypass model has been described in detail in Chapter 2. In this study, the piglets received two four hour infusions of omega-3 fatty acids: one immediately pre-operatively, and one 24 hours pre-operatively. The observation period was 24 hours following the re-institution of cardiopulmonary bypass, with intensive monitoring of cardiac, pulmonary and renal parameters as described in detail in Chapter 2. In addition, specific elements of the inflammatory response were measured to elucidate the effects of omega-3 fatty acids on the SIRS. White cell counts were measured at baseline and then at 15 minutes, 30 minutes, 1, 6, 12, 18 and 24 hours following reperfusion. The pro-

inflammatory cytokines IL-6 and IL-8 and the anti-inflammatory IL-10 were assayed on serum samples at various timepoints using porcine specific commercially available ELISA kits. Leukotriene B_4 was also assayed using a multi-species ELISA kit. NFkB was measured in harvested tissue samples using a commercially available kit suitable for porcine samples.

The pattern of injury produced in this model over the 24 hour period of observation has been described in detail in Chapter 3. Thus, in this chapter, emphasis is on the differences in results between the control animals and those pre-treated with omega-3 fatty acids.

5.3 Results:

Cardiac, pulmonary and renal function were measured as previously described over the 24 hour period of observation, with similar patterns observed between the control and omega-3 groups and consistent with the previous 8 hour study. However, although trends in improvement could be appreciated on the graphs of the clinical results in the omega-3 animals compared to the controls, the differences were not statistically significant on formal testing, with the exception of the renal and cerebral NIRS readings which were improved in the omega-3 group. The limitation in terms of the clinical results is likely related to the small numbers of the study group and thus a lack of statistical power to detect small differences.

Significant beneficial immunomodulation was observed with a decrease in pro-inflammatory cytokines and an increase in anti-inflammatory cytokines, and a reduction in levels of the pro-inflammatory eicosanoids LTB_4.

5.3.1 Cardiac Injury

Ventricular function:

Ventricular function was assessed as previously described with the Millar cardiac catheter. There was no significant difference between the control and omega-3 groups over the study period. This is seen on the graph below.

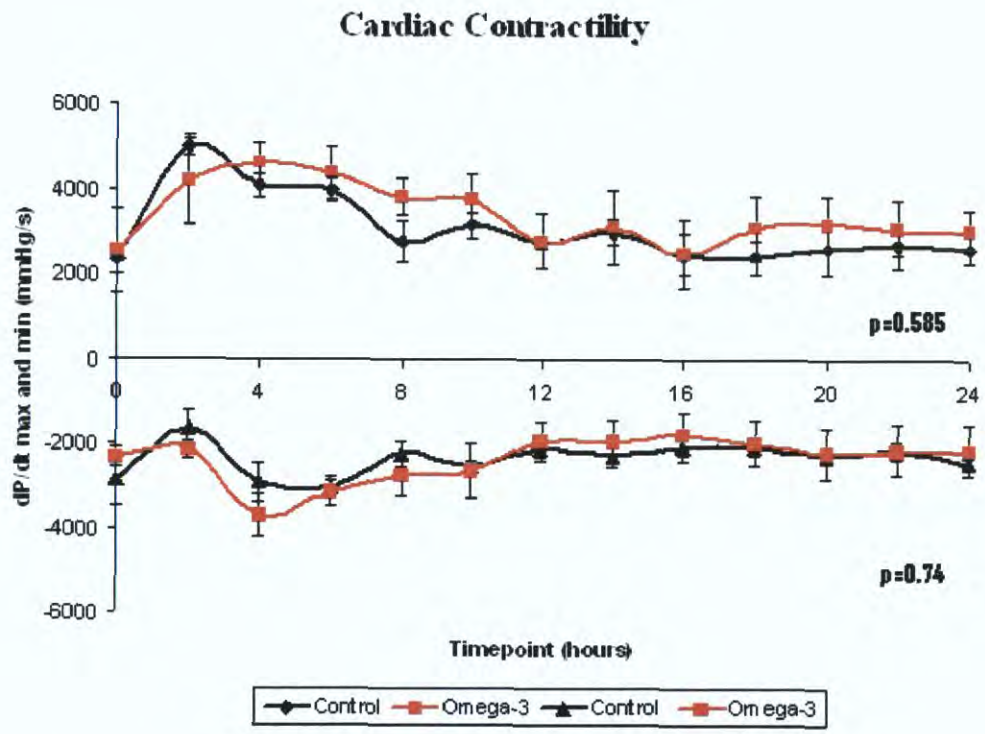

Figure 5.1

A cardiac catheter trace was recorded at baseline and two hourly thereafter. The upper graph represents systolic function (dP/dt max, mmHg), the lower graph represents diastolic function (dP/dt min, mmHg). Results are reported as mean +/-

SEM. Statistical analysis was with repeated measures ANOVA; the p value shown on the graph (Greenhouse-Geisser adjustment) represents the time vs group interaction, that is whether there is any difference in trend over time between the control and omega-3 groups; this was not significant. Group effect, testing the overall difference between the groups (i.e. the difference in means aggregated over time), was also non-significant: p=0.494.

Troponin:

Troponin T levels were measured by the Biochemistry Department in Beaumont Hospital at baseline, then at 3 hours, 6 hours, 12 hours, 18 hours and 24 hours post reperfusion. There was no significant difference between the control and omega-3 groups over the period of the study. The results are graphed below.

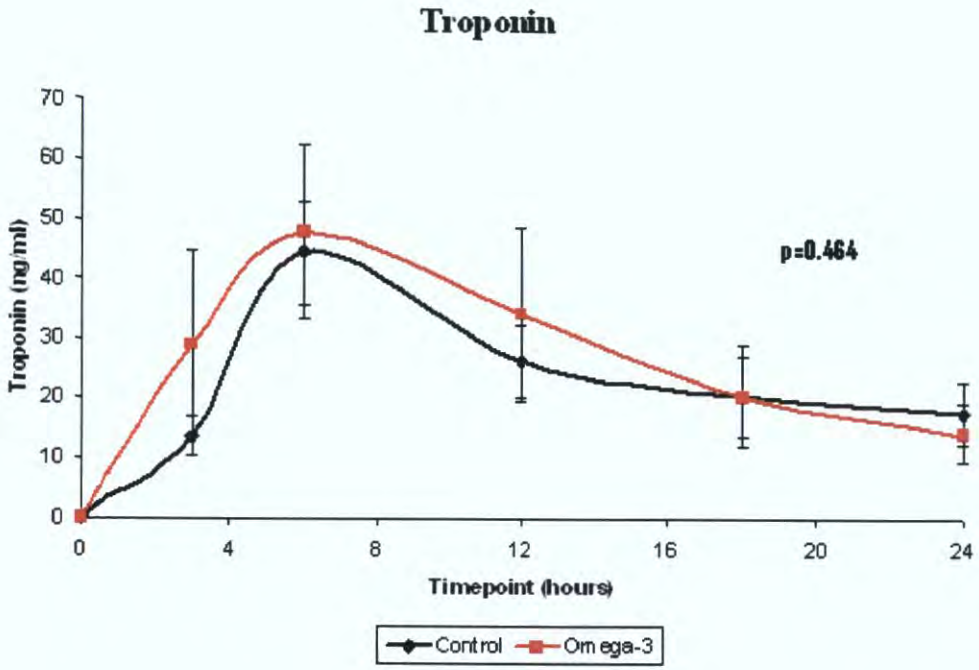

Figure 5.2

Troponin was measured at baseline, 3, 6, 12, 18 and 24 hours. Results are reported as mean troponin (ng/ml) +/- SEM. Statistical analysis was with repeated measures ANOVA; the p value shown on the graph (Greenhouse-Geisser adjustment) represents the time vs group interaction, that is whether there is any difference in trend over time between the control and omega-3 groups; this was not significant. Group effect, testing the overall difference between the groups (i.e. the difference in means aggregated over time), was also non-significant: p=0.72.

Wet:Dry Ratio:

The wet:dry ratio of harvested cardiac tissue was assessed as a measure of oedema. There was no significant difference between the groups at 24 hours.

Results are reported as mean ratio +/- SEM. Statistical analysis was with the paired t test.

Control: 4.52 +/- 0.14 Omega-3: 4.17 +/- 0.18

P=0.146 (paired t test)

Histology:

Histological examination was performed on H&E stained sections of the harvested left ventricle at 24 hours according to the scoring system shown in the table below. On examination, the changes previously observed at 8 hours had resolved, with essentially normal appearance of the tissue in all animals, and no significant differences between the groups. The composite injury score is reported below and representative images are shown in Chapter 3.

Heart	
Myocytes	
	Necrosis
	Degeneration
Interstitium	
	Inflammatory cells
	Oedema
	Haemorrhage
Pericardium	
	Inflammatory cells
	Oedema
	Haemorrhage
Vessels	
	Endothelial activation

	Obliteration/thrombosis
	Vasculitis

Table 1: Histological scoring system for the analysis of the H&E stained cardiac sections. The slides were scored on a severity score of 0 to 3 by a blinded pathologist. A composite histological injury score was thus obtained and used for comparison between the control and omega-3 groups.

Composite histological injury score:

Results are reported as mean score +/- SEM. Statistical analysis was with a paired t test.

Control: 1.8 +/- 0.2 Omega-3: 1.8 +/- 0.7

P=0.5 (paired t test)

5.3.2 Development of a low cardiac output state

Hemodynamic measurements were recorded hourly: heart rate, mean arterial blood pressure and central venous pressure. The patterns in the control animals were described in detail in Chapter 3. These patterns were consistent in the omega-3 animals, and there were no statistically significant differences between the control and omega-3 groups. These results are shown in the following graphs.

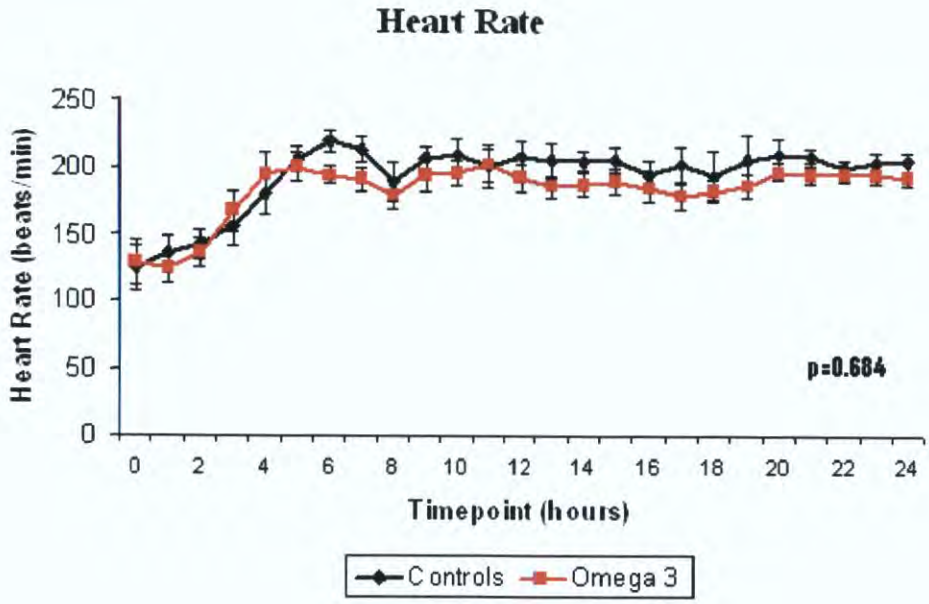

Figure 5.3

Heart rate was recorded hourly. Results are reported as mean heart rate (beats per minute) +/- SEM. Statistical analysis was with repeated measures ANOVA; the p value shown on the graph (Greenhouse-Geisser adjustment) represents the time vs group interaction, that is whether there is any difference in trend over time between the control and omega-3 groups; this was not significant. Group effect, testing the overall difference between the groups (i.e. the difference in means aggregated over time), was also non-significant: p=0.326.

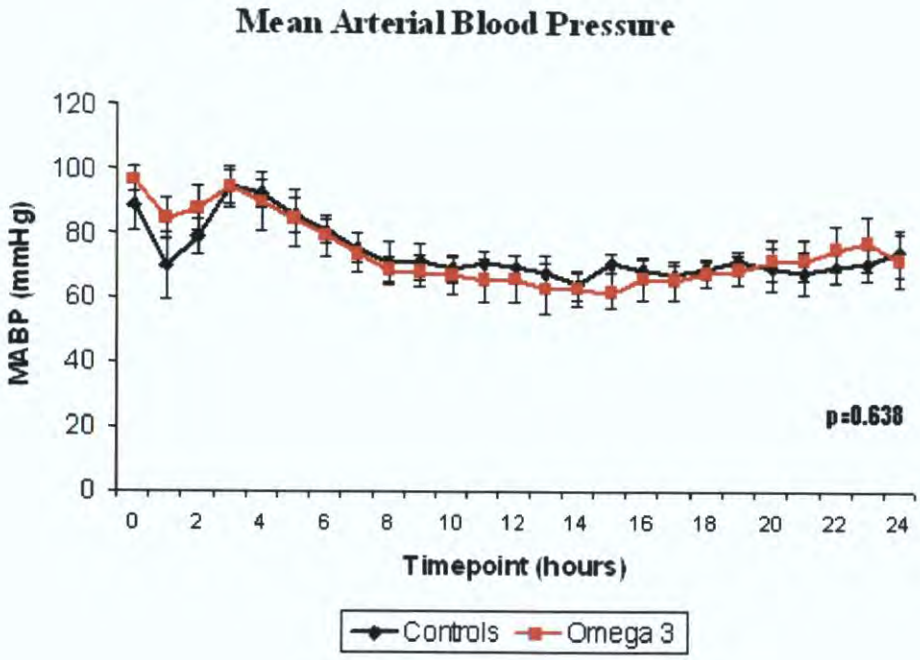

Figure 5.4

Mean arterial blood pressure was recorded hourly from the femoral arterial line. Results are reported as mean MABP (mmHg) +/- SEM. Statistical analysis was with repeated measures ANOVA; the p value shown on the graph (Greenhouse-Geisser adjustment) represents the time vs group interaction, that is whether there is any difference in trend over time between the control and omega-3 groups; this was not significant. Group effect, testing the overall difference between the groups (i.e. the difference in means aggregated over time), was also non-significant: p=0.978. Although there were no significant differences between the groups, from the graph, the early drop in MABP in the first hour does appear attenuated in the omega-3 group.

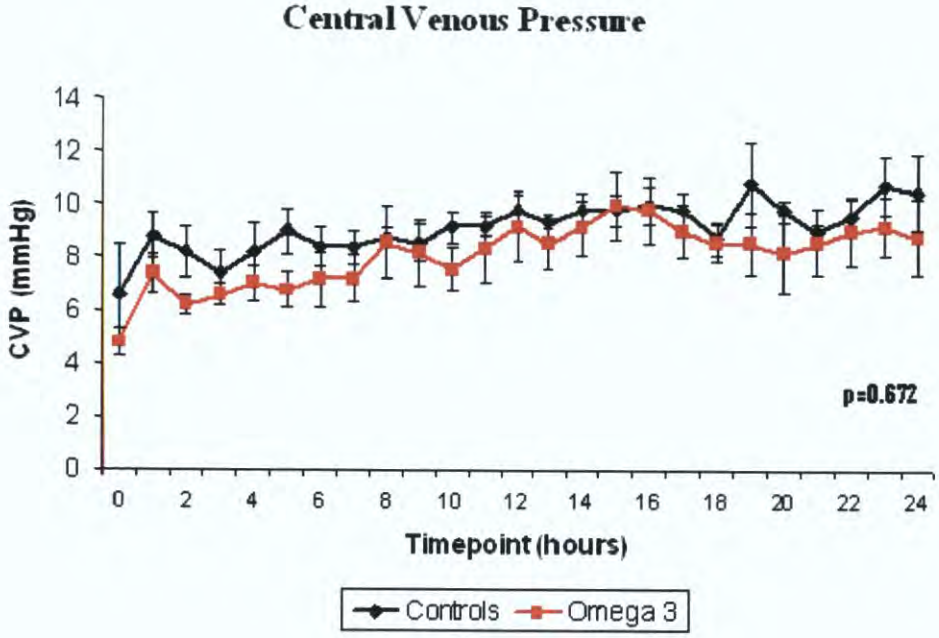

Figure 5.6

Central venous pressure was recorded hourly from the femoral venous line. Results are reported as mean CVP (mmHg) +/- SEM. Statistical analysis was with repeated measures ANOVA; the p value shown on the graph (Greenhouse-Geisser adjustment) represents the time vs group interaction, that is whether there is any difference in trend over time between the control and omega-3 groups; this was not significant. Group effect, testing the overall difference between the groups (i.e. the difference in means aggregated over time), was also non-significant: p=0.652.

As measures of tissue perfusion, lactate, base excess, mixed venous oxygen saturations, and renal and cerebral regional oxygen saturations were recorded regularly. The patterns observed over the 24 hours of observation were similar between the two groups. There were no statistically significant differences between the control and omega-3 groups with regard to lactate, base excess and mixed venous oxygen saturations, although the graph of

mixed venous oxygen saturations does trend towards improved readings in the omega-3 group. However, there was a definite trend towards improved renal cortical NIRS readings, and cerebral NIRS readings were significantly improved. This is indicative of improved perfusion of the renal and cerebral cortices and thus an attenuation of the low cardiac output syndrome observed in the very early post reperfusion period and from approximately 8 – 10 hours onwards, as described in Chapter 3. These results are shown in the graphs below.

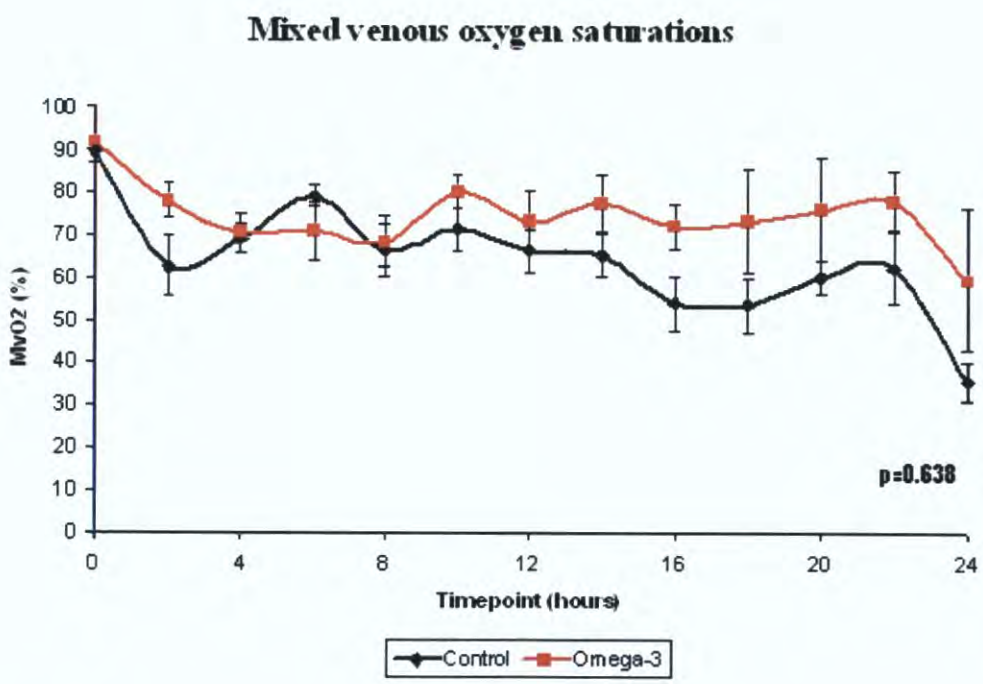

Figure 5.7

Mixed venous oxygen saturations were measured every two hours on central venous blood gas analysis. Results are reported as mean MvO_2 (%) +/- SEM. Statistical analysis was with repeated measures ANOVA; the p value shown on the graph

(Greenhouse-Geisser adjustment) represents the time vs group interaction, that is whether there is any difference in trend over time between the control and omega-3 groups; this was not significant. Group effect, testing the overall difference between the groups (i.e. the difference in means aggregated over time), was also non-significant: p=0.112.

Although MvO_2 was not significantly different between the groups on formal testing, from the graph, the early reduction in MvO_2 seen in the control animals appears attenuated and the omega-3 values do appear higher from approximately 8 hours onwards.

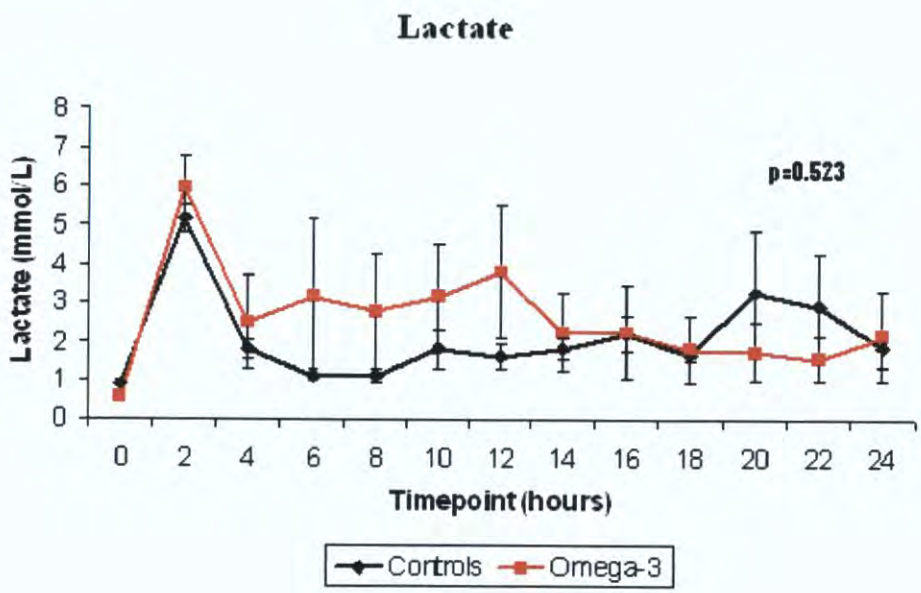

Figure 5.8

Lactate was recorded two hourly from arterial blood gas analysis. Results are reported as mean lactate (mmol/L) +/- SEM. Statistical analysis was with repeated

measures ANOVA; the p value shown on the graph (Greenhouse-Geisser adjustment) represents the time vs group interaction, that is whether there is any difference in trend over time between the control and omega-3 groups; this was not significant. Group effect, testing the overall difference between the groups (i.e. the difference in means aggregated over time), was also non-significant: p=0.484.

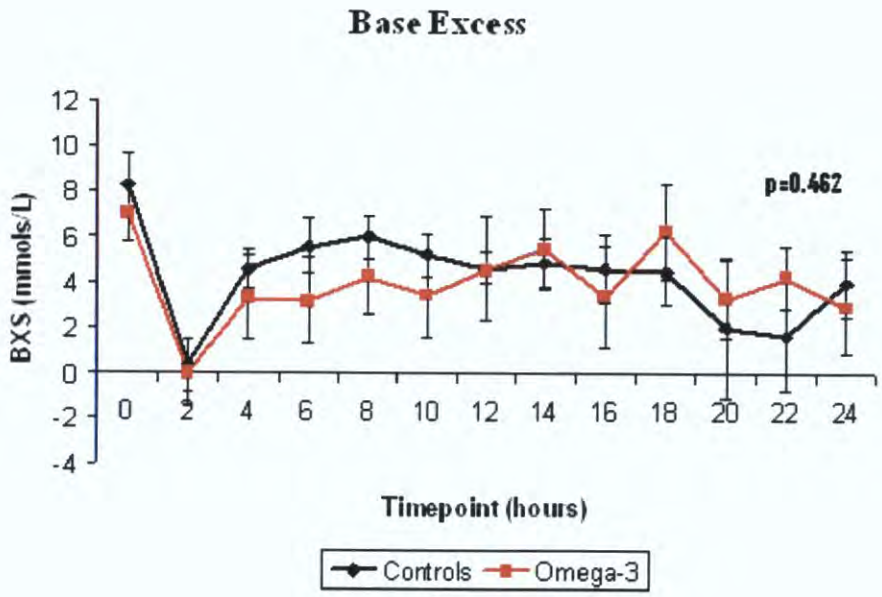

Figure 5.9

Base excess was recorded two hourly from arterial blood gas analysis. Results are reported as mean base excess (mmols/L) +/- SEM. Statistical analysis was with repeated measures ANOVA; the p value shown on the graph (Greenhouse-Geisser adjustment) represents the time vs group interaction, that is whether there is any

difference in trend over time between the control and omega-3 groups; this was not significant. Group effect, testing the overall difference between the groups (i.e. the difference in means aggregated over time), was also non-significant: p=0.564.

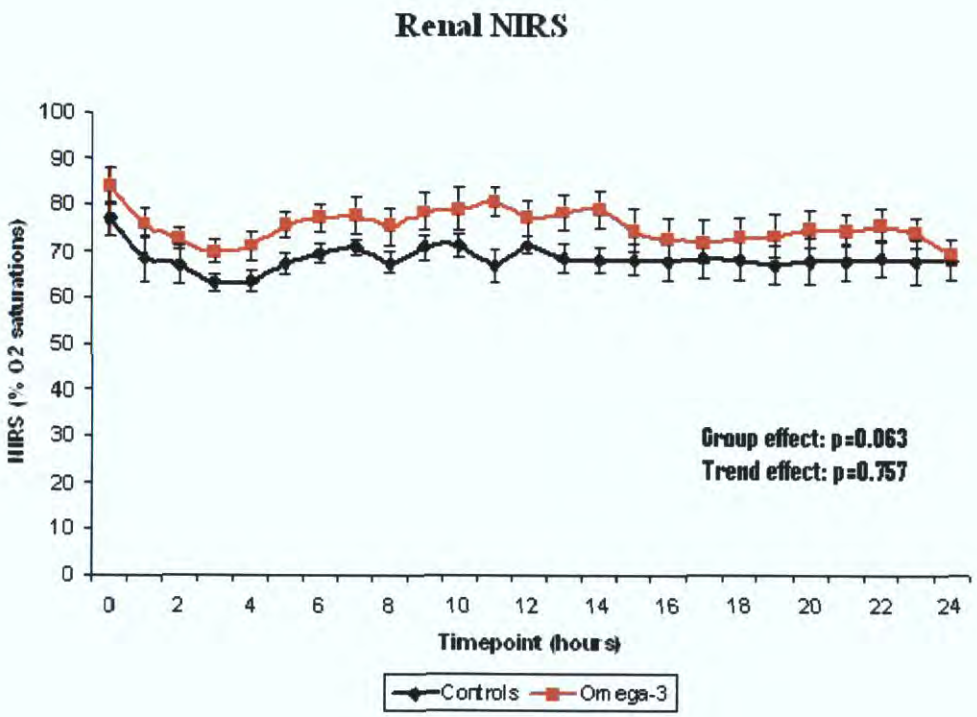

Figure 5.10

Regional renal oxygen saturations using NIRS were recorded hourly. Results are reported as mean +/- SEM. Statistical analysis was with repeated measures ANOVA; the trend effect p value shown on the graph (Greenhouse-Geisser adjustment) represents the time vs group interaction, that is whether there is any difference in trend over time between the control and omega-3 groups; this was not significant. Group effect, testing the overall difference between the groups (i.e. the

difference in means aggregated over time), was also non-significant: p=0.063. However, this does represent a definite trend towards an overall improvement in renal NIRS with omega-3 pre-treatment.

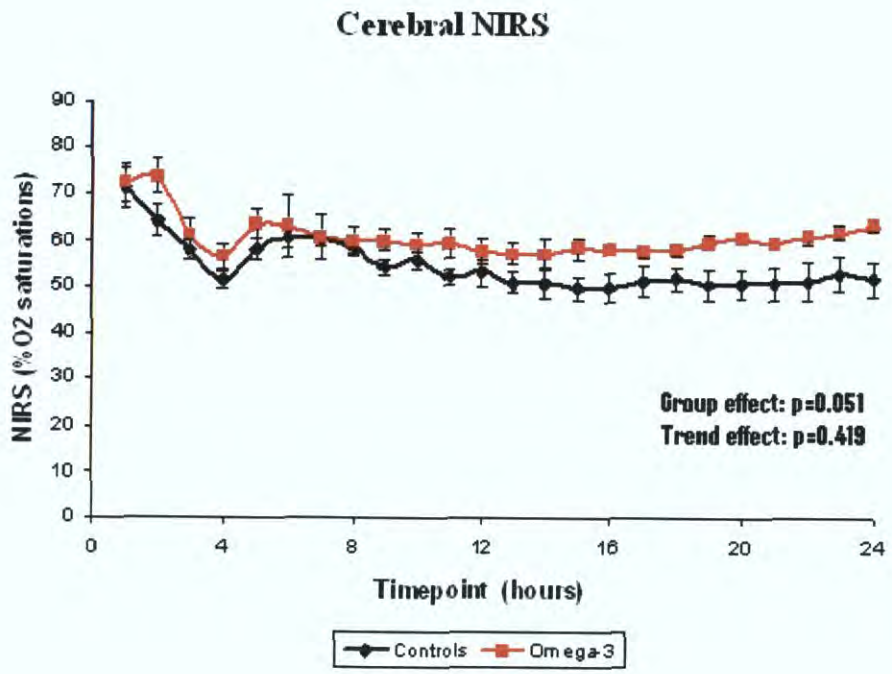

Figure 5.11

Cerebral regional oxygen saturations using NIRS were recorded hourly. Results are mean +/- SEM. Statistical analysis was with repeated measures ANOVA; the trend effect p value shown on the graph (Greenhouse-Geisser adjustment) represents the time vs group interaction, that is whether there is any difference in trend over time between the control and omega-3 groups; this was not significant. However, the

group effect testing for cerebral NIRS was effectively significantly increased in the omega-3 group, p=0.051.

As factors affecting hemodynamic readings and tissue perfusion, temperature and hematocrit were recorded. There were no significant differences between the groups as shown below.

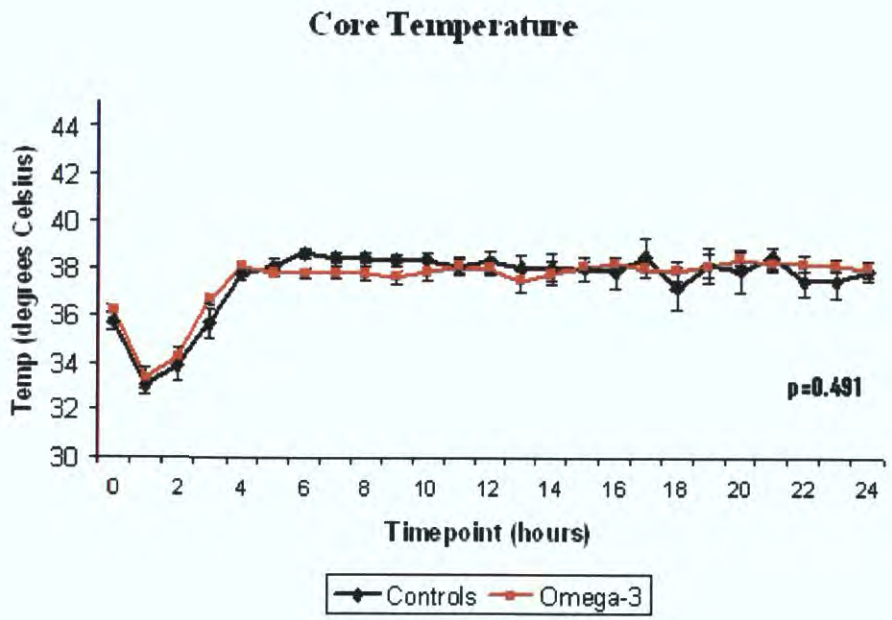

Figure 5.12

Core temperature was measured from a rectal probe and recorded hourly. Results are reported as mean temperature (°C) +/- SEM. Statistical analysis was with repeated measures ANOVA; the p value shown on the graph (Greenhouse-Geisser adjustment) represents the time vs group interaction, that is whether there is any difference in trend over time between the control and omega-3 groups; this was not

significant. Group effect, testing the overall difference between the groups (i.e. the difference in means aggregated over time), was also non-significant: p=0.979.

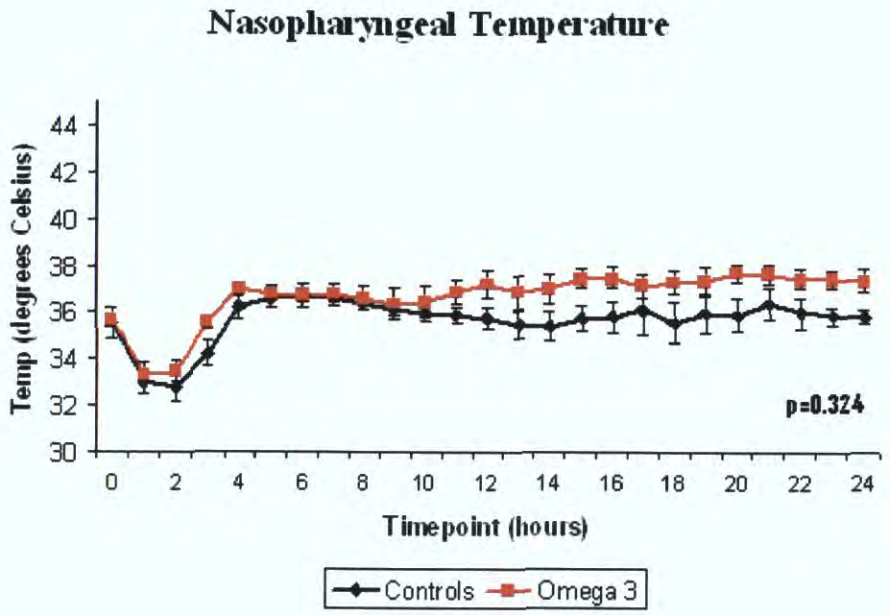

Figure 5.13

Nasopharyngeal temperature was recorded hourly. Results are reported as mean temperature (^{o}C) +/- SEM. Statistical analysis was with repeated measures ANOVA; the p value shown on the graph (Greenhouse-Geisser adjustment) represents the time vs group interaction, that is whether there is any difference in trend over time between the control and omega-3 groups; this was not significant. Group effect, testing the overall difference between the groups (i.e. the difference in means aggregated over time), was also non-significant: p=0.081.

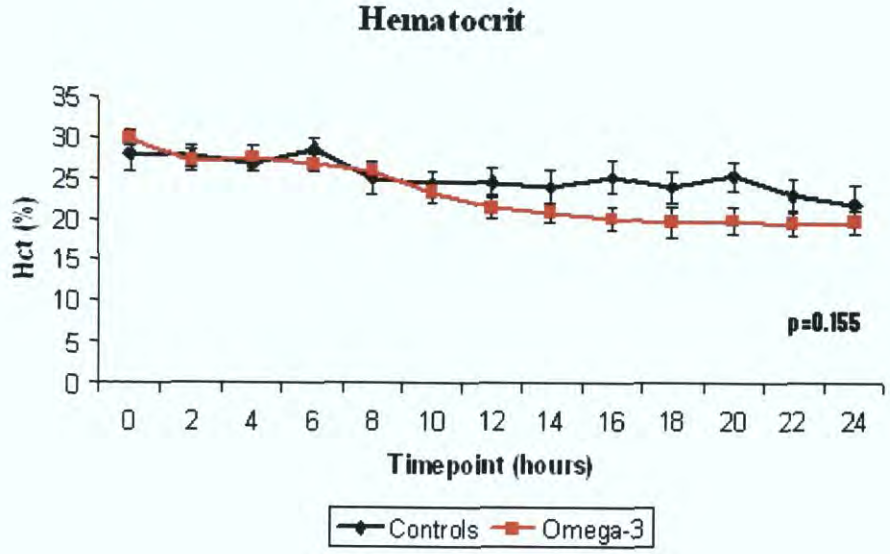

Figure 5.14

Hematocrit was recorded every two hours from the arterial blood gas analysis. Results are reported as mean hematocrit (%) +/- SEM. Statistical analysis was with repeated measures ANOVA; the p value shown on the graph (Greenhouse-Geisser adjustment) represents the time vs group interaction, that is whether there is any difference in trend over time between the control and omega-3 groups; this was not significant. Group effect, testing the overall difference between the groups (i.e. the difference in means aggregated over time), was also non-significant: p=1.213.

5.3.2 Pulmonary Injury

Pulmonary Compliance:

Pulmonary compliance was recorded hourly from the CO_2SMO Plus respiratory profile monitor. An improvement at 8 hours in both static and dynamic compliance was seen in the 8 hour study. However, over the 24 hour period of observation, there was no significant difference overall in dynamic compliance between the two groups as shown on the graph below. (There was insufficient data to analyze static compliance due to technical issues).

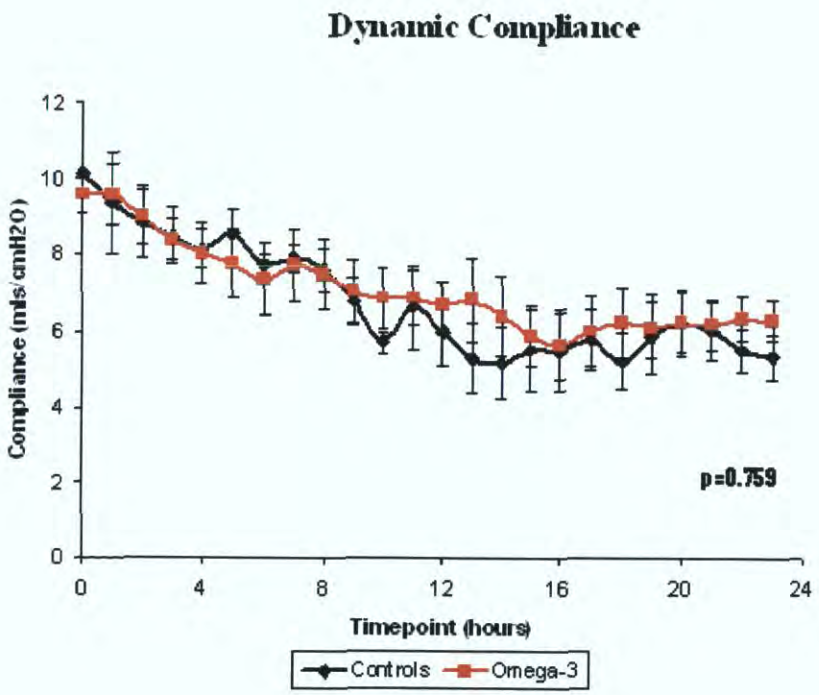

Figure 5.15

Dynamic compliance was recorded hourly. Results are reported as mean compliance (mls/cmH_20) +/- SEM. Statistical analysis was with repeated measures ANOVA; the p value shown on the graph (Greenhouse-Geisser adjustment) represents the time vs group interaction, that is whether there is any difference in trend over time between the control and omega-3 groups; this was not significant. Group effect, testing the overall difference between the groups (i.e. the difference in means aggregated over time), was also non-significant: p=0.913.

Airway Resistance:

As another measure of the mechanics of ventilation, inspiratory and expiratory airway resistance was also recorded from the CO_2SMO Plus respiratory profile monitor. However, due to technical difficulties not all recordings were complete, and therefore there were insufficient data points for formal statistical analysis of these parameters. The graphs are displayed below, and there does appear to be a trend towards less resistance to ventilation in the omega-3 pre-treatment animals.

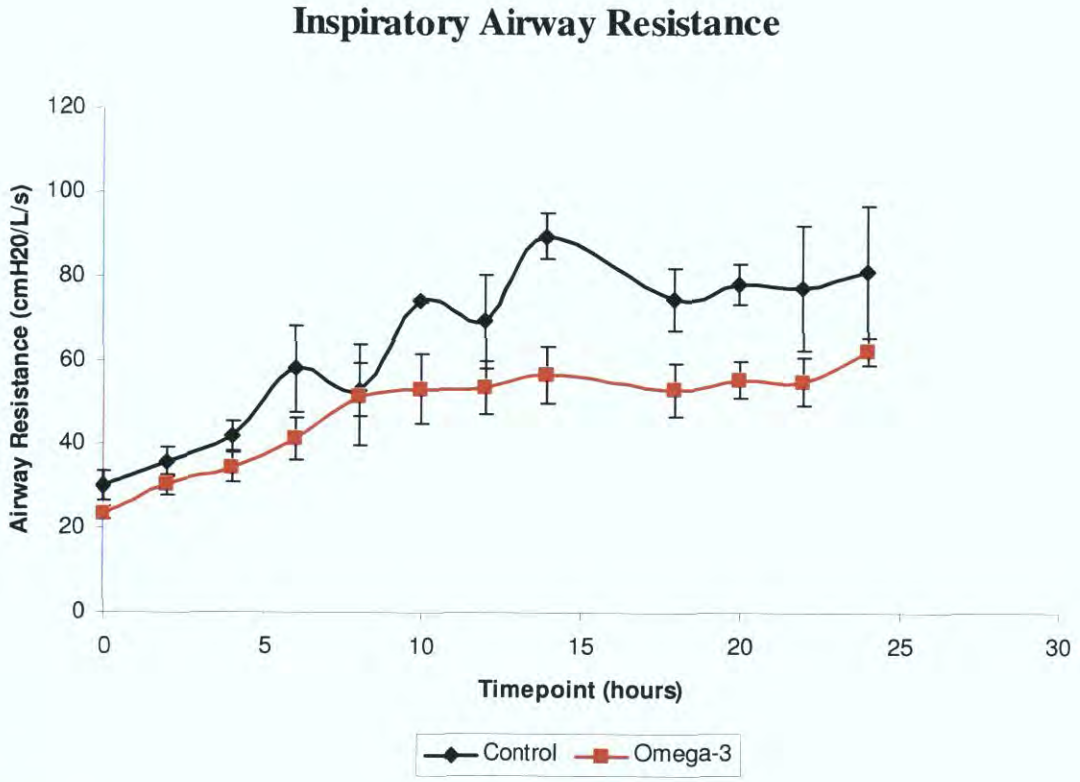

Figure 5.16

Inspiratory airway resistance was recorded two hourly. Results are reported as mean resistance ($cmH_20/L/s$) +/- SEM. There was insufficient data for formal statistical analysis, however there does appear to be a trend towards improved readings in the omega-3 group.

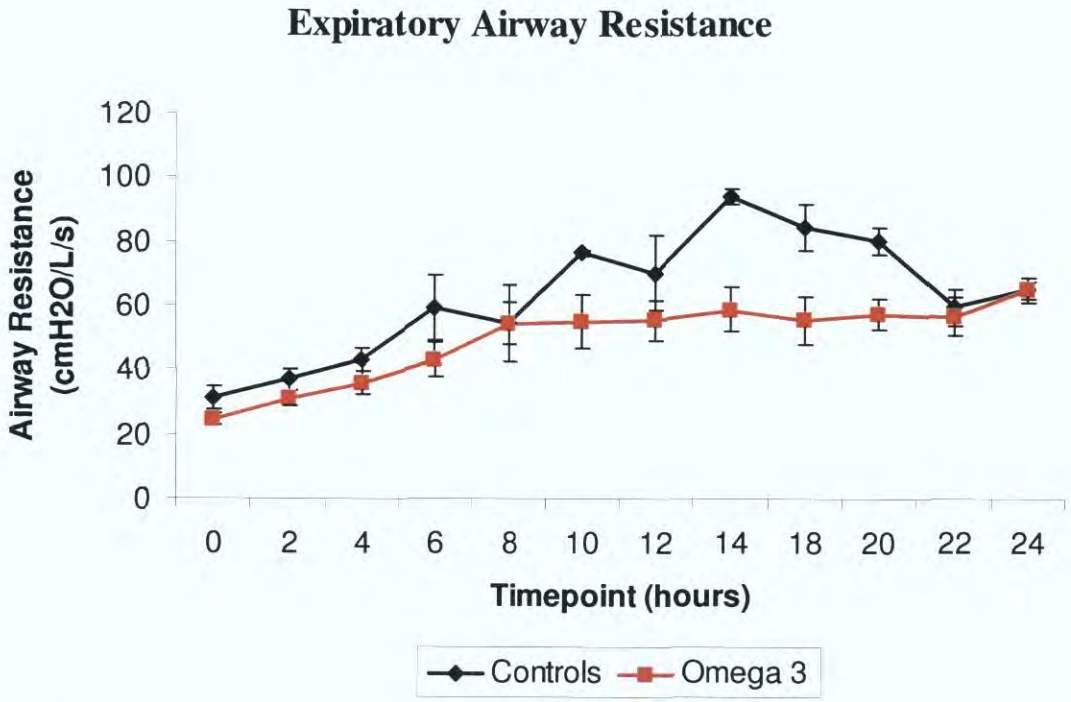

Figure 5.17

Expiratory airway resistance was recorded two hourly. Results are reported as mean resistance ($cmH_20/L/s$) +/- SEM. There were insufficient data points for formal statistical analysis; however, the results from the omega-3 group trend towards an improvement in airway resistance.

Alveolar:arterial gradient:

As a marker of gas exchange, the alveolar:arterial gradient was calculated from the arterial blood gas analysis every two hours. There was no statistically significant difference with omega-3 pre-treatment as shown on the graph below.

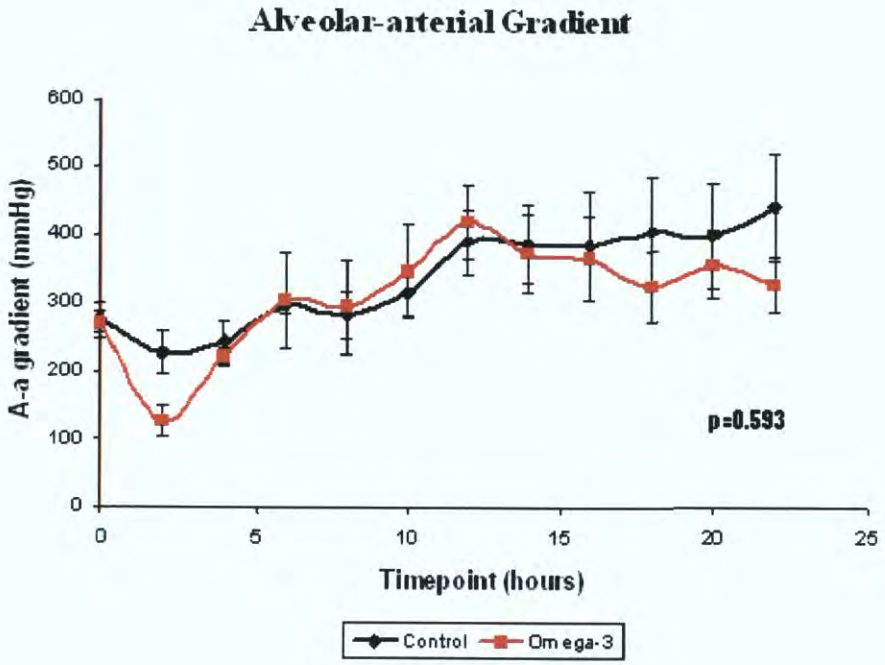

Figure 5.18

A-a gradient was calculated every two hours from the arterial blood gas analysis. Results are reported as mean gradient (mmHg) +/- SEM. Statistical analysis was with repeated measures ANOVA; the p value shown on the graph (Greenhouse-Geisser adjustment) represents the time vs group interaction, that is whether there is any difference in trend over time between the control and omega-3 groups; this was not significant. Group effect, testing the overall difference between the groups (i.e. the difference in means aggregated over time), was also non-significant: p=0.67.

Partial Pressure of Oxygen (pO_2):

The pO_2 was recorded as a measure of oxygenation. There was no statistically significant difference between the groups, although there is a trend towards improvement in the omega-3 pre-treated group from approximately 16 hours onwards.

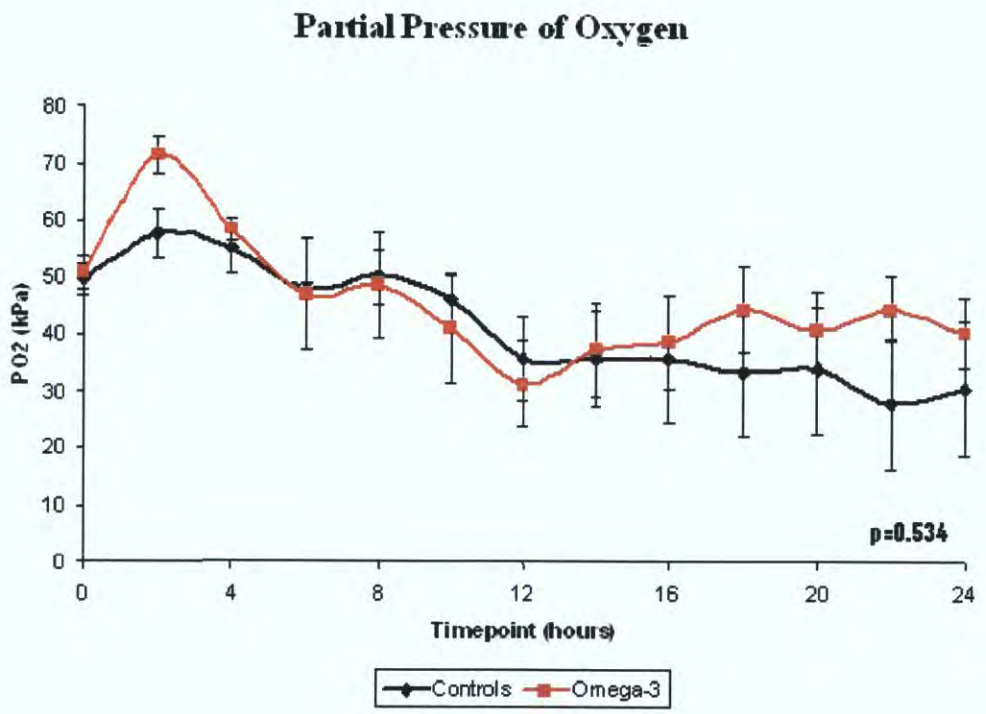

Figure 5.19

The pO2 was recorded two hourly from arterial blood gas analysis. Results are reported as mean +/- SEM. Statistical analysis was with repeated measures ANOVA; the p value shown on the graph (Greenhouse-Geisser adjustment) represents the time vs group interaction, that is whether there is any difference in trend over time between the control and omega-3 groups; this was not significant.

Group effect, testing the overall difference between the groups (i.e. the difference in means aggregated over time), was also non-significant: 0.578.

Although the difference was not significant, there does appear to be a trend towards increased pO_2 in the omega-3 group from 14 – 16 hours onwards.

Wet:Dry Ratio:

The wet:dry ratio was measured as an indication of tissue oedema. There was no significant difference between the two groups at 24 hours. Results are reported as mean ratio +/- SEM. Statistical analysis was with the paired t test.

Controls: 7.21 +/- 0.45 Omega-3: 6.41 +/- 0.43

P=0.17 (paired t test)

Histology:

Histological analysis was performed on the harvested sample of the left lower lobe. The parameters assessed are shown in the table below. At the end of the 24 hour period of observation, histological examination of the harvested lung tissue was returning to normal in all animals with no significant differences between the groups. The composite injury scores are shown below and representative images are shown in Chapter 3.

Lung	
Alveoli	
	Inflammatory cell infiltration
	Oedema
	Haemorrhage
Interstitium	

	Congestion
	Inflammatory cell infiltration
	Oedema
	Thickening
Pleura	
	Inflammatory cell infiltration
	Oedema
	Haemorrhage
Vessels	
	Endothelial activation
	Obliteration/thrombosis
	Vasculitis

Table 2: Histological features assessed on examination of the H&E stained sections of lung tissue. Each parameter was scored from 0 to 3 by a blinded pathologist and the composite injury score used to compare between the groups.

Composite injury scores:

Results are reported as mean +/- SEM. Statistical analysis was with the paired t test.

Controls: 6.8 +/- 2.2 Omega-3: 4.4 +/- 1

P=0.087

5.3.4 Renal injury:

Renal injury was assessed using the hourly urine output; renal NIRS as a measure of renal cortical perfusion; creatinine clearance as a measure of glomerular function; and urinary N-acetyl-glucosaminidase and fractional excretion of urinary sodium as measures of tubular structure and function respectively. There were no statistically significant differences in any of the renal parameters measured. However, in the omega-3 group as

discussed above, the renal NIRS readings did trend towards an improvement in the omega-3 group indicating improved perfusion. Also, hourly urine output did appear to trend towards an earlier recovery above 1ml/kg/hr from approximately 18 hours onwards in the omega-3 group compared to controls; similarly, the fractional excretion of urinary output trended towards an earlier recovery from approximately 16 hours onwards. The urinary NAG levels, although not statistically significantly different over the whole period of observation, did trend towards an increase in the omega-3 group, particularly at 2 hours post reperfusion. This result was unexpected. Studies with steroids or off-pump studies which lead to a reduction in the inflammatory response have been shown to reduce the levels of urinary NAG following cardiac surgery. It is possible that this result is due to an inaccuracy in the assay or due to the low numbers of the study.

The renal results are graphed below.

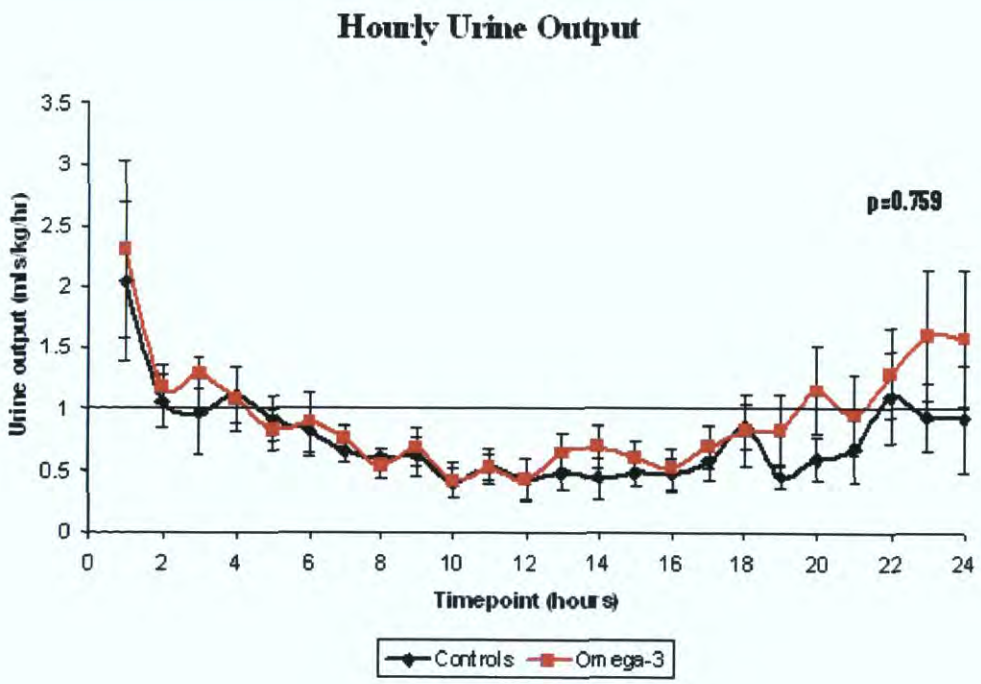

Figure 5.20

Urine output was measured hourly from the suprapubic catheter. Results are reported as mean urine output per kilogram body weight per hour +/- SEM. Statistical analysis was with repeated measures ANOVA; the p value shown on the graph (Greenhouse-Geisser adjustment) represents the time vs group interaction, that is whether there is any difference in trend over time between the control and omega-3 groups; this was not significant. Group effect, testing the overall difference between the groups (i.e. the difference in means aggregated over time), was also non-significant: p=0.372.

Although there were no statistically significant differences between the control and omega-3 groups, there does appear from the graph to be an earlier recovery of urine output to above 1ml/kg/hr from approximately 18 hours onwards.

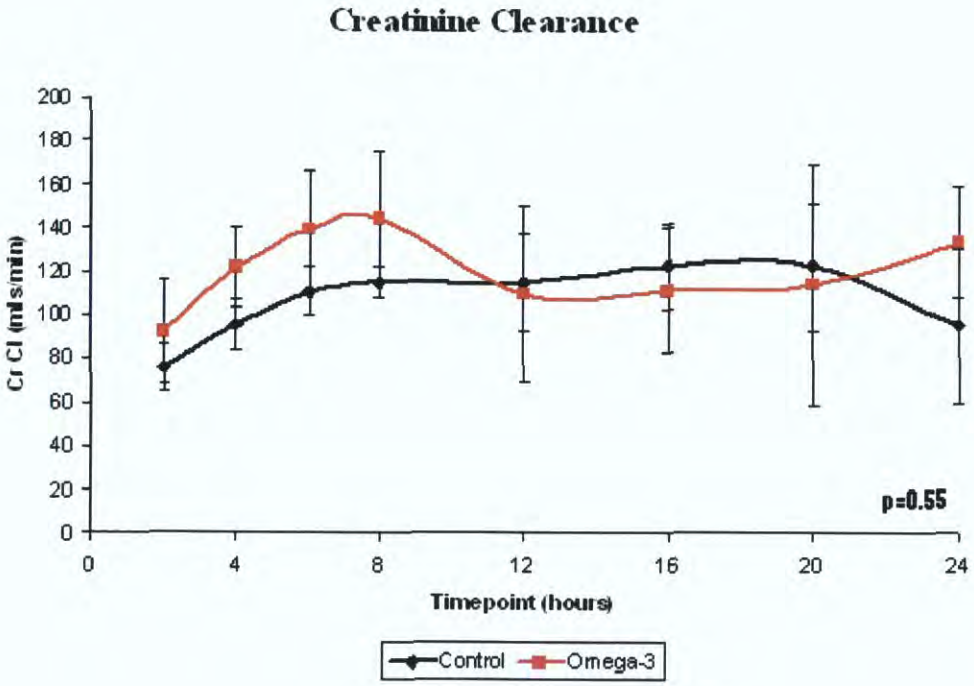

Figure 5.21

Creatinine clearance was recorded every two hours as a marker of glomerular function. Results are reported as mean +/- SEM. Statistical analysis was with repeated measures ANOVA; the p value shown on the graph (Greenhouse-Geisser adjustment) represents the time vs group interaction, that is whether there is any difference in trend over time between the control and omega-3 groups; this was not significant. Group effect, testing the overall difference between the groups (i.e. the difference in means aggregated over time), was also non-significant: p=0.732.

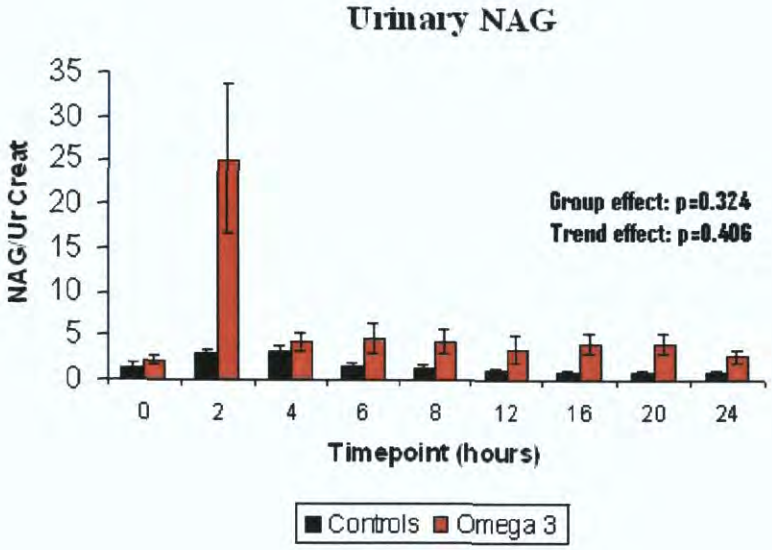

Figure 5.22

Urinary NAG was assayed at baseline and every two hours as a marker of tubular structural damage. Results are reported as mean +/- SEM. Statistical analysis was with repeated measures ANOVA; the p value shown on the graph (Greenhouse-Geisser adjustment) represents the time vs group interaction, that is whether there is any difference in trend over time between the control and omega-3 groups; this was not significant. Group effect, testing the overall difference between the groups (i.e. the difference in means aggregated over time), was also non-significant: p=0.324.

Although the differences over time were not significantly different between the groups on formal testing, the two hour peak does appear higher in the omega-3 group.

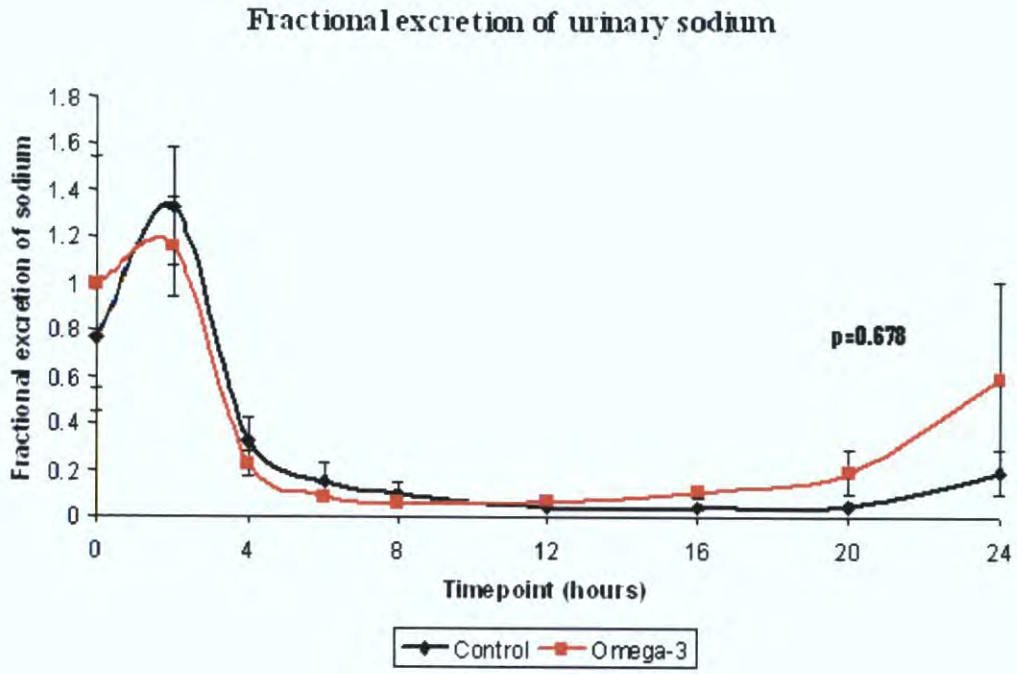

Figure 5.23

Fractional excretion of urinary sodium was calculated every two hours. Results are reported as mean fraction +/- SEM. Statistical analysis was with repeated measures ANOVA; the p value shown on the graph (Greenhouse-Geisser adjustment) represents the time vs group interaction, that is whether there is any difference in trend over time between the control and omega-3 groups; this was not significant. Group effect, testing the overall difference between the groups (i.e. the difference in means aggregated over time), was also non-significant: p=0.709.

Wet:Dry Ratio:

The wet:dry ratio was measured as an indication of tissue oedema. There was no significant difference in oedema at 24 hours. Results are reported as mean +/- SEM. Statistical analysis was with paired t test.

Controls: 5.5 +/- 0.41 Omega-3: 7.24 +/- 1.17

P=0.221 (paired t test)

Histology:

Histological examination of the harvested renal tissue at 24 hours demonstrated no significant pathological changes in any of the samples; there were no significant differences between the omega-3 and control groups. The scoring system used and the composite injury scores are shown below. Representative images are shown in Chapter 3.

Kidney	
Interstitium	
	Inflammatory cell infiltration
	Oedema
Vessels	
	Endothelial activation
	Obliteration/thrombosis
	Vasculitis
Glomeruli	
	Inflammatory cell infiltration
Tubules	
	Debris
	Cytoplasmic vacuolation

Table 3: Histological scoring system for the renal H&E stained sections. Each parameter was scored from 0 to 3 in severity by a blinded pathologist and the composite injury score used for comparison between the groups.

Composite injury score:

Results are reported as mean +/- SEM. Statistical analysis was with paired t test.

Control: 1.8 +/- 0.5 Omega-3: 0.8 +/- 0.2

P=0.071 (paired t test)

5.3.5 Mechanism of Action of Omega-3:

In order to investigate the mechanism of action of omega-3 fatty acids, certain elements of the SIRS were measured. This included white cell counts at baseline, 15 minutes, 30 minutes, 1 hour, 6 hours, 12 hours, 18 hours and 24 hours following reperfusion. This was performed by the Biochemistry Laboratory in Beaumont Hospital. The results did not demonstrate any significant differences on formal statistical testing over the measured timepoints. However, at 24 hours, the omega-3 group trended towards a higher WCC compared to controls. This may represent a reduction in immunosuppression at this time.

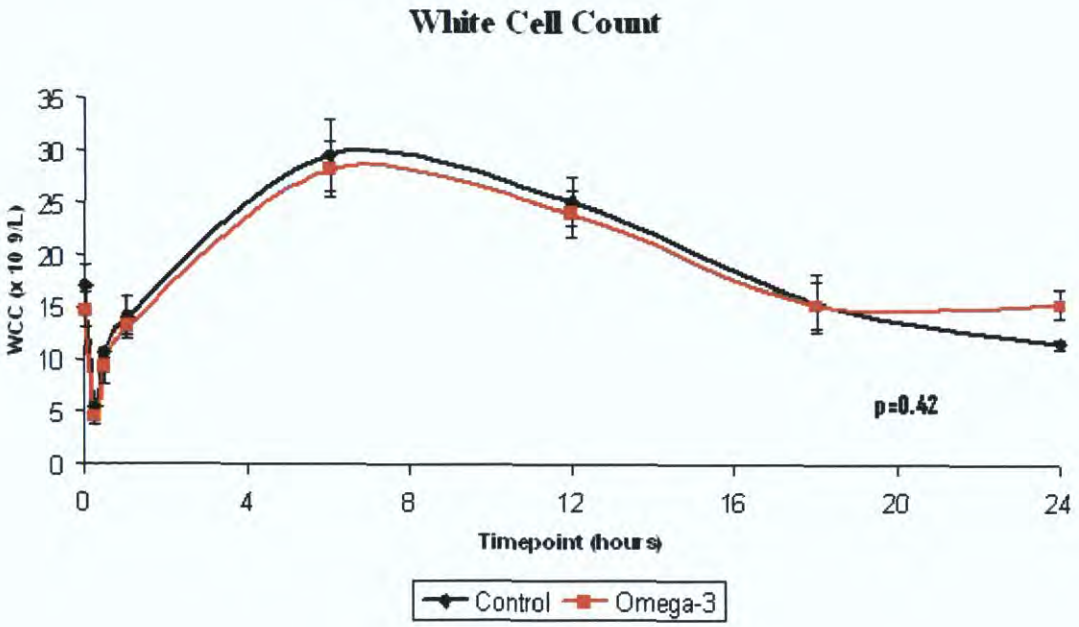

Figure 5.24

White cell counts were measured by the Biochemistry Laboratory. Results are reported as mean +/- SEM. Statistical analysis was with repeated measures ANOVA; the p value shown on the graph (Greenhouse-Geisser adjustment) represents the time vs group interaction, that is whether there is any difference in trend over time between the control and omega-3 groups; this was not significant. Group effect, testing the overall difference between the groups (i.e. the difference in means aggregated over time), was also non-significant: p=0.916.

As markers of the inflammatory response, the pro-inflammatory cytokines IL-6 and IL-8, and the anti-inflammatory cytokine IL-10 were measured using porcine specific

commercially available ELISA kits. A trend towards reduced IL-6 levels at four hours was evident on the graph, however this was not statistically significant. A significant reduction in IL-8 levels at two hours was demonstrated in the omega-3 animals. A significant increase in the level of IL-10 over the 24 hour period was also noted. This is indicative of an attenuation of SIRS, through a decrease in pro-inflammatory and an increase in anti-inflammatory cytokines. These results are illustrated in the following graphs.

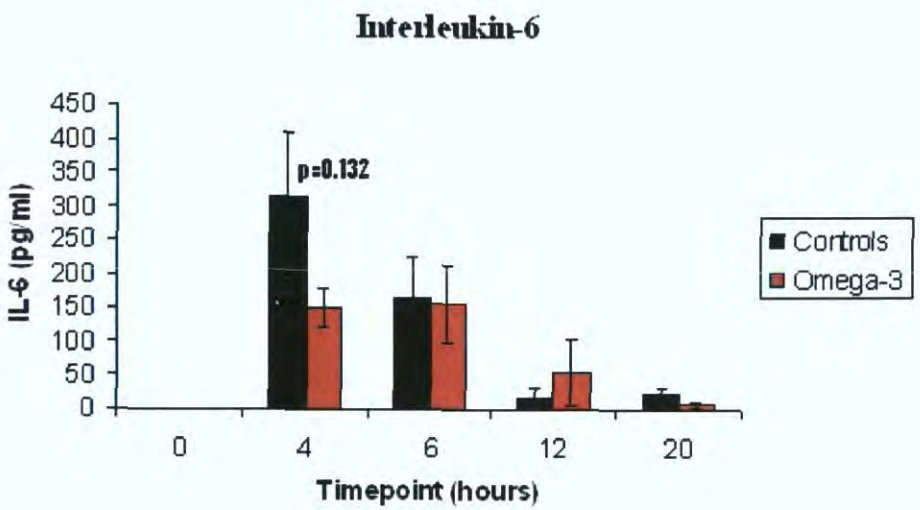

Figure 5.25

Interleukin-6 was measured using a porcine specific ELISA. Results are reported as mean IL-6 +/- SEM. Statistical analysis was with repeated measures ANOVA; the p value shown on the graph compares the 4 hour values only – this was not significant.

The group effect (testing the overall difference between the groups) and the time vs group interaction tests (testing the difference in trends between the groups) did not reach statistical significance: p=0.459 and p=0.175 respectively. There was a trend towards a reduction in IL-6 levels at 4 hours on the graph, however this was not statistically significant.

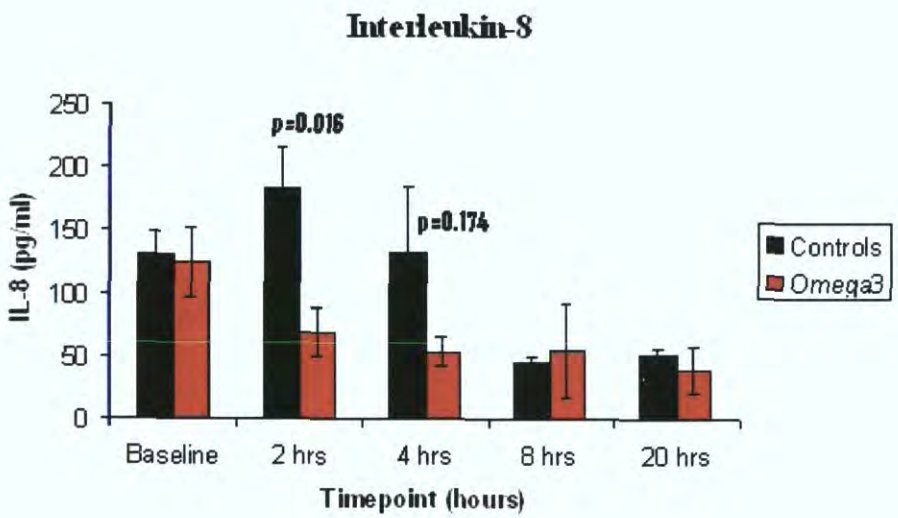

Figure 5.26

Interleukin-8 was measured with a porcine specific ELISA. Results are reported as mean IL-8 +/- SEM. Statistical analysis was with repeated measures ANOVA. The p values on the graph are the comparisons at the 2 and 4 hour timepoints only. The evident reduction was significant only at 2 hours, although a trend is appreciated on

the graph at 4 hours also. The overall group test and time vs group interaction tests were not statistically significant: p=0.208 and p=0.067 respectively.

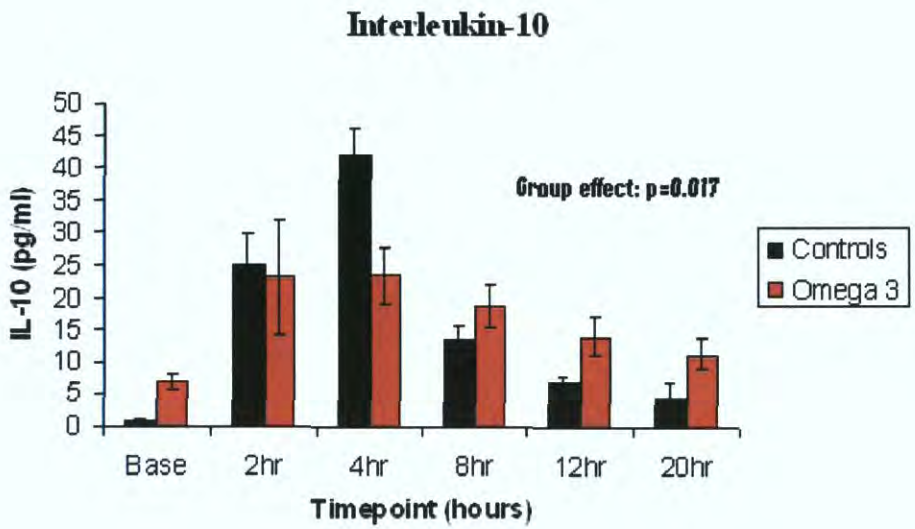

Figure 5.27

Interleukin-10 was measured using a porcine specific ELISA. Results are reported as mean IL-10 +/- SEM. Statistical analysis was with repeated measures ANOVA. The group effect p value shown on the graph represents the overall difference between the group, i.e. there is a significant increase in IL-10 in the omega-3 group overall. The time vs group interaction (testing the trends between the group) was no significant: p=0.443.

The effects of omega-3 on the inflammatory system have been attributed both to an increased ratio of omega-3 to omega-6 fatty acids in cell membranes which leads to antagonism of the production of arachidonic acid derived mediators, and direct actions on intracellular signaling pathways which lead to reduced activation of transcription factors such as NFkB. Both of these pathways were investigated by measuring Leukotriene B4 and NFkB.

In response to an inflammatory stimulus, phospholipases cleave arachidonic acid (omega-6) or EPA (omega-3) from cell membrane phospholipids and release them as free fatty acids. These are then converted into eicosanoids: the prostanoids and the leukotrienes. Importantly, the eicosanoids produced from the conversion of EPA are anti-inflammatory (prostanoids of the 3 series and leukotrienes of the 5 series), while those derived from AA are pro-inflammatory (prostanoids of the 2 series, leukotrienes of the 4 series). By increasing the ratio of EPA to AA in the cell membranes, production of the AA derived eicosanoids will be decreased and the EPA eicosanoids increased. LTB_4 is easily measured with a commercially available ELISA kit – this is a multispecies kit and therefore suitable for porcine samples. A significant reduction in the levels of LTB_4 was observed in animals pretreated with omega-3. This is demonstrated on the graph below.

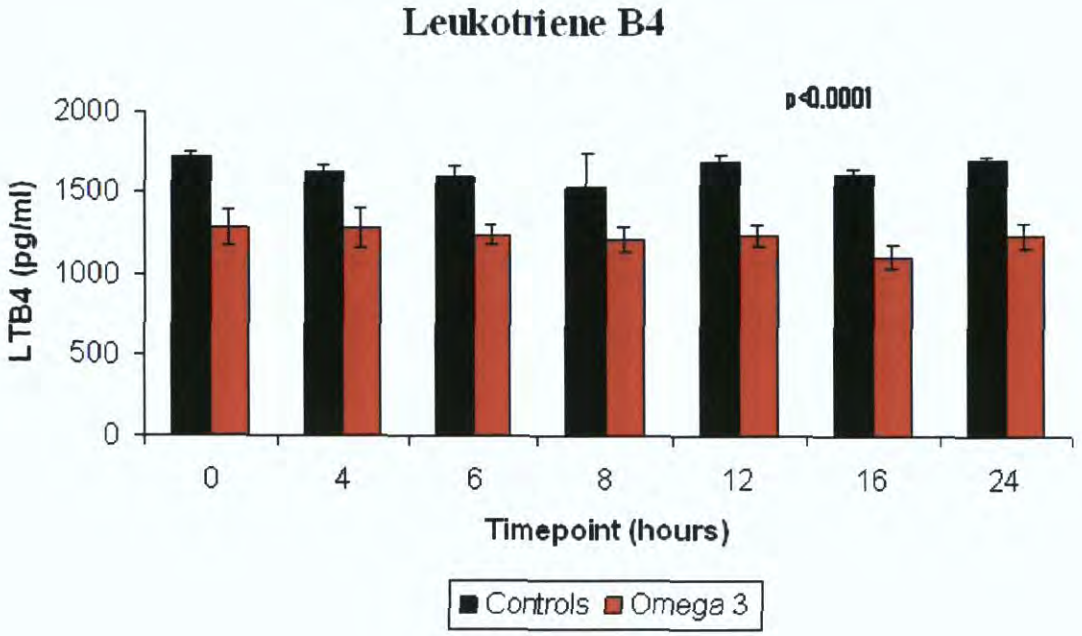

Figure 5.28

LTB_4 was measured at baseline and then at 4, 6, 8, 12, 16 and 24 hours following reperfusion using a multispecies ELISA. Results are reported as mean LTB_4 (pg/ml) +/- SEM. Statistical analysis was with repeated measures ANOVA, the group effect p value (representing the overall difference between the groups) is statistically significant at $p<0.0001$. The time vs group interaction was not significant: $p=0.603$. Thus LTB_4 was reduced at all timepoints in the omega-3 pre-treatment groups.

As the major transcription factor involved in the inflammatory response, we measured NFkB levels in all the major organs at 24 hours. It is important to remember that the levels of NFkB were compared at baseline, 8 and 24 hours in the control animals in

Chapter 2 – a reduction from baseline in all organs was observed at 8 hours; in the lung, the levels appeared lower again at 24 hours; while in the heart and kidney, levels appeared to increase from 8 to 24 hours (however, these changes were not statistically significant). This graph is included here also to allow for comparison.

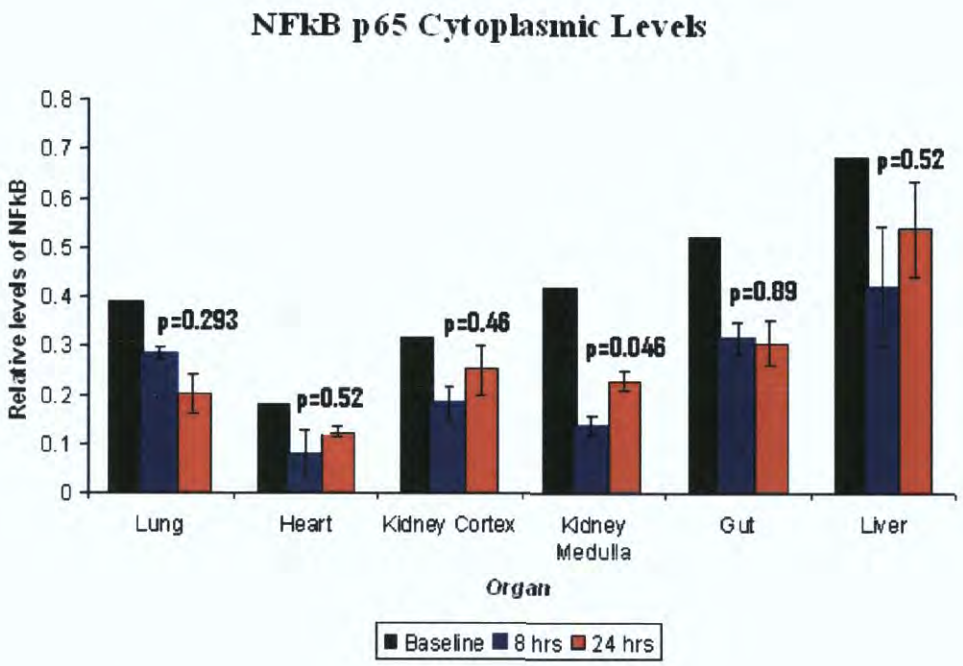

Figure 5.29

NFkB levels were measured at 8 and 24 hours in our control animals and compared to a sham animal as a baseline level. Results are reported as mean relative levels of NFkB +/- SEM. Levels were reduced from baseline in all organs at 8 hours. At 24 hours, levels in the lung appeared to have decreased further, while levels in the heart and kidney appeared to have increased compared to the 8 hour values. However, formal statistical analysis with independent samples t tests and

Bonferroni correction of significance level to account for the number of tests performed (therefore significance level for these tests is p<0.0042) did not demonstrate any significant differences between the levels at 8 and at 24 hours in this control group.

Levels of NFkB were then measured in the 24 hour omega-3 group in order to compare the levels with those of the control animals. NFkB expression appeared to be increased in the omega-3 animals compared to controls in all organs, however this was not statistically significant.

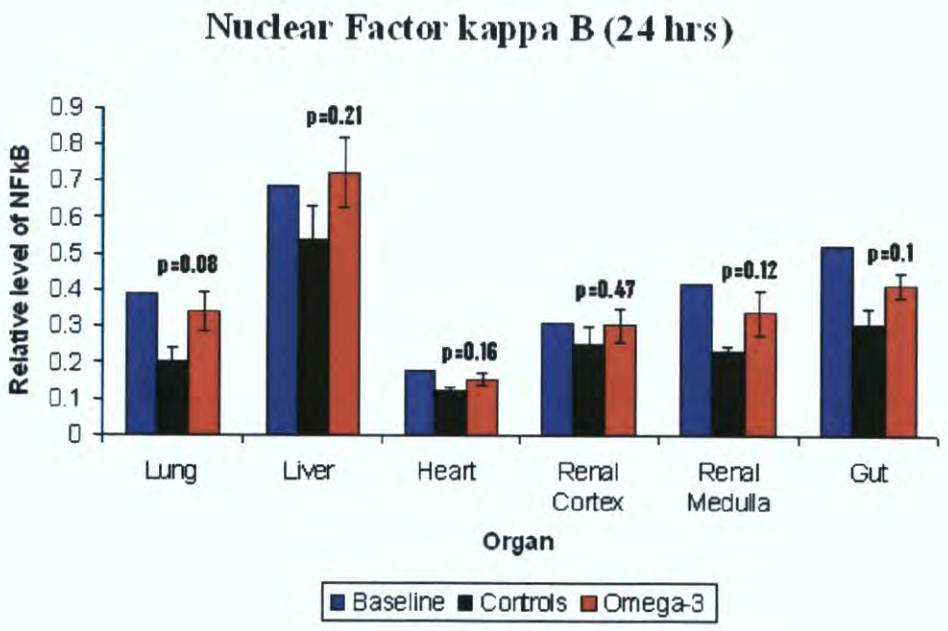

Figure 5.30

NFkB was measured in organ tissue samples at 24 hours. Results are reported as mean relative level of NFkB +/- SEM. Statistical analysis was with independent sample t tests, with Bonferroni correction of the significance level to account for the number of comparisons made (therefore, the significance level for these tests is $p<0.0042$). The omega-3 group trends towards higher NFkB expression in all organs particularly in the lungs.

This finding of a trend towards increased NFkB levels in the omega-3 group compared to controls is the opposite to what would be expected given that omega-3 has been previously shown to exert its anti-inflammatory effects through reducing the activation of NFkB. However, when compared to the baseline values, we see that at 24 hours, the NFkB levels in the omega-3 group are not higher than baseline values. Therefore, this increase represents a more rapid return to baseline values, which may be indicative of a reduction in the immunosuppression normally seen at this time following cardiac surgery.

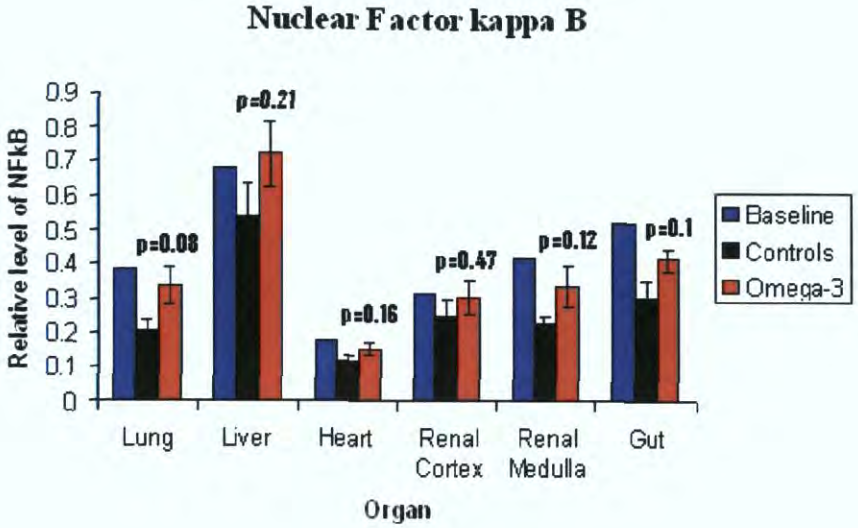

Figure 5.31

NFkB levels in the control and omega-3 groups at 24 hours compared to baseline levels. Results are reported as mean +/- SEM. Statistical analysis was with the independent samples t test to compare the omega-3 vs control groups at 24 hours. Although there was no significant differences between the groups, the graph does show a trend of NFkB levels returning to baseline in the omega-3 group while they *remain lower in the control group.*

5.4 Discussion:

As the initial study conducted using the juvenile piglet model with survival to eight hours post-operatively demonstrated improved cardiopulmonary function with omega-3 pretreatment, the aim of this study, which has an increased period of observation and a second pre-operative dose of omega-3 fatty acids, was to determine if these early results

were sustained and led to further improvements in pulmonary indices and an attenuation of the low cardiac output demonstrated in the control animals.

As described in detail in Chapter 3, two periods of low cardiac output syndrome were observed in this model: one immediately post reperfusion until 4 hours post-operatively (low diastolic blood pressure in the first hour, reduced mixed venous oxygen saturations, and reduced renal and cerebral NIRS readings); and a second period, which was noticeable from approximately 8 hours onwards (initial low diastolic blood pressure from 7 hours with MABP reduced from 8 hours, cerebral NIRS readings low from 8 hours, metabolic acidosis from 18-20 hours). The initial period of low cardiac output is normally attributable to myocardial stunning[4]; however, in this study, left ventricular function was not impaired. Early temperatures were low, therefore an increased peripheral vascular resistance at this time may have contributed to this. The second period of low cardiac output is most likely accounted for by vasomotor dysfunction secondary to the SIRS. Omega-3 pretreatment did not have any statistically significant protective effects on the pulmonary or renal parameters measured. With regard to the cardiac parameters, there were no significant improvements in left ventricular function or troponin levels. However, perfusion was improved as indicated by an overall increase in both renal and cerebral NIRS readings; in addition mixed venous oxygen saturations trended towards an improvement from approximately 8 hours onwards. Thus an attenuation of the low cardiac output seen in the control animals is evident. The important limiting factor in this study is the small numbers and thus a lack of statistical power to detect small differences.

Therefore, it would be hoped that with a larger clinical trial, statistically significant improvements in these parameters would be apparent.

In order to examine the mechanism of action of omega-3 fatty acids, a number of measurements of the SIRS were measured. White cell counts were not different between the groups over the 24 hours. This was in contrast to the 8 hour study conducted (Chapter 4) in which omega-3 animals demonstrated significantly reduced white cell count levels at 6 hours post reperfusion. The most likely confounder in these findings is the relatively small numbers in both studies. There are no studies which detail the acute numbers of neutrophils following an inflammatory stimulus in response to omega-3 pre-treatment. However, as a reduction in the SIRS is expected, a reduction in WCC would have been anticipated at this timepoint. The trend towards a late increase in WCC 24 hours post reperfusion in the omega-3 group is likely to represent a preservation of immune function at this stage; that is an avoidance of the period of immuno-suppression usually seen following surgery. Animal studies have demonstrated increased neutrophils at 16 hours following inflammatory stimulation with omega-3 pre-treatment in a mouse model of pseudomonas aeruginosa infection[5]; in addition, omega-3 pre-treatment in a rat model of sepsis prevented the sepsis-induced suppression of lymphocyte proliferation at 24 hours[6]. In post-operative gastrointestinal cancer patients, enteral feeding with an omega-3 supplemented diet resulted in higher levels of total lymphocytes, T lymphocytes, and TH cells[7].

With regard to the anti-inflammatory actions of omega-3 fatty acids on cytokines, a reduction in IL-6 and IL-8, and an increase in IL-10 were noted in our pre-treated animals. This is a trend frequently observed with other strategies which attenuate the immune response such as steroids[8,9]. Omega-3 fatty acids have been shown to attenuate the production of IL-6 and IL-8 both in vitro[10] and in vivo[11,12,13]; and to increase levels of IL-10[14],[15]. In addition, they have been shown to attenuate the leucocyte-endothelial interactions central to both ischemia-reperfusion injury and SIRS, through their inhibitory action on adhesion molecule expression[4,16]. It would have been interesting in this study to measure levels of ICAM-1 or E-Selectin; however, there are no specific porcine ELISA kits available, and due to the poor amino acid homology (<50%) between swine and human ICAM-1 and E-Selectin, the human kits available for this purpose were not suitable.

The anti-inflammatory effects of omega-3 fatty acids also extend to the reduction of pro-inflammatory eicosanoids derived from arachidonic acid and the concomitant increase in the production of anti-inflammatory eicosanoids derived from EPA in response to an inflammatory stimulus[17,18]. This is achieved with supplementation through the increased incorporation of EPA and DHA into the phospholipids of cell membranes, thus increasing the omega-3/omega-6 fatty acid ratio[19,20]. Leukotriene B_4 is one of the arachidonic acid derived eicosanoids. It is a potent neutrophil chemoattractant[21], and also has a role in the production of inflammatory cytokines[22] and vascular permeability[23]. In this study, a significant reduction in the level of LTB_4 was observed at baseline throughout the 24 hour period following cardiopulmonary bypass. The limitation here is

that an eicosanoid derived from EPA such as Leukotriene B_5 was not assayed for comparison; an increase in the level of LTB_5 would have been expected as has been demonstrated in previous studies[8,9].

As described, there is a potent pro-inflammatory response following cardiac surgery, which is inhibited by omega-3 fatty acids. However, at the same time as the induction of inflammation, anti-inflammatory cytokines are also induced in order to naturally limit this reaction, thus leading to a period of immuno-suppression following cardiac surgery[24]. This period appears to occur at approximately 24 hours following cardiac surgery, with a reduction in the ability of immune cells to respond to a further inflammatory stimulus[25], and a shift in the TH1/TH2 balance in favour of the anti-inflammatory TH2[26]. This alteration in balance between T helper cells has been shown to predispose to post-operative infection in colorectal cancer patients[27]. Omega-3 fatty acids have been shown to ameliorate this immunosuppression in both animal and clinical studies by increasing the TH1/TH2 ratio, increasing TH1 cytokine production and improving the phagocytic activity of macrophages[28,29], effects important in responding particularly to infection. In examining the NFkB findings in this study, it was noted that the omega-3 pre-treated animals had a return to baseline levels of NFkB at 24 hours compared to controls in whom levels remained depressed at this time. This may represent an important action of omega-3 in attenuating the immunosuppression seen at this time following cardiopulmonary bypass, thereby allowing a more normal stress response to a further insult such as infection. It would be interesting in this model to examine the TH1/TH2

balance at this time point to see if omega-3 has had a favourable anti-inflammatory effect on this balance, in addition to the improved NFkB levels.

Recent studies have also focused on the importance of omega-3 fatty acids in the resolution of inflammation through the production of lipid mediators from EPA and DHA via the lipoxygenase and cyclo-oxygenase 2 pathways: resolvins (resolution-phase interaction products), and docosatrienes and neuroprotectins (specific dihydroxy acid containing docosatrienes) [30,31,32]. Resolvins have been shown to reduce neutrophil infiltration in a murine model of peritonitis[33]; and to reduce pro-inflammatory gene expression and prevent the development of experimental colitis in mice[34]. With the same mechanism, 10, 17S-docosatriene (also called neuroprotectin D1) has demonstrated protection against brain ischemia-reperfusion mediated leukocyte infiltration with consequent reduction of stroke infarct volume in a mouse model[35]. Thus, these mediators represent an additional pathway for the protective effects of omega-3 fatty acids. Currently measurement of these mediators is with liquid chromatography-mass spectroscopy (LC-MS) and is technically challenging and quite expensive; as such, this was not possible at present in this model. Efforts to produce an ELISA are underway, but this is not yet available.

In summary therefore, the protective effects of omega-3 fatty acids occur through a number of pathways: a decrease in pro-inflammatory cytokines and an increase in anti-inflammatory cytokines; an increase in the omega-3/omega-6 ratio in cell membrane phospholipids resulting in a shift towards the inhibition of pro-inflammatory eicosanoids

and an increase in anti-inflammatory eicosanoids; an attenuation of the post-operative period of immuno-suppression; and the production of newly described lipid mediators important in the resolution of inflammation – resolvins and protectins. In this study, a number of these effects were demonstrated: decreased levels of IL-6 and IL-8, and increased IL-10; a reduction in the production of LTB_4 (a pro-inflammatory eicosanoid); and trends in WCC and NFkB indicative of a reduction in post-operative immunosuppression. Further work in this model to fully elucidate the effects of omega-3 fatty acids following paediatric cardiac surgery could include the measurement of additional eicosanoids, a determination of the TH1/TH2 ratio of lymphocytes at 24 hours, and the measurement of resolvins and protectins. In addition, it would be interesting to measure the levels of membrane fatty acids – previous work by McGuinness et al demonstrated a 15-fold increase in EPA and a 7-fold increase in DHA in myocardial cell membranes in a rabbit model with four days of pre-treatment with omega-3 fatty acids[1].

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CHAPTER 6:

DISCUSSION:

Paediatric cardiac surgery is a dynamic specialty in which major advances have been made in the last thirty years. The first successful arterial switch operation was performed in 1975 by Dr Adib Jatene[1]; while in 1983, Norwood reported the first successful staged palliative reconstructive operations for hypoplastic left heart syndrome[2], until which time this condition had been universally fatal[3]. Since then, rapid improvements in the mortality rates following these procedures have occurred. The Hospital for Sick Children in Toronto reported hospital survival following the Norwood procedure of 41% from 1990 – 1993; 61% from 1994 – 1997; and 81% from 1998 – 2000[4]. Similar improvements have been reported in the literature by other institutions[3]. With these improvements in mortality, attention has now focused on reducing the significant morbidity encountered in these patients. A degree of cardiac, pulmonary and renal dysfunction are frequently seen post-operatively in these patients; this may be mild or transient, however in some this dysfunction can progress to organ failure. Multiple system organ failure is a severe complication, with a high mortality rate and the possibility of long-term sequelae[5]. Much research has been undertaken to determine the cause of organ dysfunction following cardiac surgery, and lately, the systemic inflammatory response has been ascribed a central role[6,7,8,9].

Cardiac surgery induces the systemic inflammatory response via three mechanisms: myocardial ischemia-reperfusion injury; gut ischemia; and contact with the

cardiopulmonary bypass circuit, which causes activation of platelets and of the complement, kinin-kallikrein, and coagulation cascades. This leads to activation of circulating inflammatory cells, neutrophils in particular, and the endothelium; cellular transcription factors such as NFkB are activated and lead to increased production of inflammatory cytokines; and leukocyte-endothelial interactions result in transmigration of neutrophils into the tissues with the consequent release of reactive oxygen species and damaging enzymes such as myeloperoxidase, elastase and metalloproteinases. These leukocyte-endothelial interactions are the key pathological process in the SIR to cardiac surgery[6-9]. In addition to activating this pro-inflammatory response which is maximal in the first 12 -24 hours post-operatively, cardiac surgery also induces anti-inflammatory strategies at the same time, such as the release of interleukin-10[10,11]. Also naturally occurring negative feedback loops are induced such as that observed with NFkB[12]. These actions result in a period of immuno-suppression following cardiac surgery occurring approximately 24 hours after surgery[13,14]. This period is therefore also a vulnerable time, during which the body's response to a further inflammatory stimulus such as infection may be impaired[14].

Several strategies have attempted to attenuate the systemic inflammatory response to cardiac surgery and bypass both in laboratory models and in clinical practice, such as steroids[15,16,17], leucocyte filtration[18,19], heparin coated bypass circuits[20], neutrophil elastase inhibitors[21] and complement inhibitors[22]. However, results are conflicting and none have consistently shown a benefit in clinical practice.

Omega-3 fatty acid intake has for many years now been associated with significant health benefits, particularly cardiovascular. This was initially recognized in epidemiological studies of the Greenland Eskimos and the people of the Mediterranean, who with a diet high in omega-3 fatty acids had a low incidence of coronary artery disease[23], and much research has been undertaken since with benefits now appreciated in primary[24] and secondary prevention of myocardial infarction[25,26], lipid profiles[27], and reduction of post-operative atrial fibrillation[28]. In addition, clinical studies in general surgical patients have demonstrated reductions in infectious complications, intensive care and overall hospital stays[29], and shortened the length of time of mechanical ventilation[30] and renal replacement therapy[31]. The mechanism of action of these beneficial effects has been attributed to the anti-inflammatory, anti-infarct, and anti-arrhythmic properties of omega-3 fatty acids[32]. Multiple pathways for these effects are recognized: a reduction of pro-inflammatory cytokines and an increase in anti-inflammatory cytokines[33]; an increase in the omega-3/omega-6 fatty acid ratio in cell membranes which results in increased anti-inflammatory eicosanoid production[34,35]; an attenuation of the period of post-operative immunosuppression[36]; production of the newly described resolvins and protectins, important in the resolution of inflammation[37,38]; and induction of preconditioning as suggested by our own endothelial and neutrophil cell work[39].

The hypothesis of this research was that a pre-operative intravenous infusion of omega-3 fatty acids in a clinically approved formulation would produce beneficial anti-inflammatory effects resulting in improved cardiac, pulmonary and renal function in the early post-operative period in a juvenile piglet model of cardiopulmonary bypass. This

was based on the previous work in our laboratory which demonstrated an attenuation of cytokine production and leucocyte endothelial activation in stimulated saphenous vein endothelial cells with omega-3 pre-treatment[39], and a reduction in myocardial infarct size in a rabbit regional ischemia –reperfusion model with omega-3 pre-treatment[40].

Two studies were conducted using a multi-organ juvenile piglet model of cardiopulmonary bypass. The first had an eight hour period of observation, and was used mainly as a preliminary study to ensure smooth running of the model, which is newly established in our laboratory, and to assess organ function post-operatively and demonstrate the early effects of omega-3 pre-treatment. The results demonstrated an improvement in pulmonary compliance at eight hours, and an attenuation of early diastolic dysfunction and as a result, we proceeded to the second, more detailed study which observed the piglets to 24 hours post-operatively. The results of the control animals from both this and the 8 hour study were combined to observe the pattern, timing and extent of organ injury post paediatric cardiac surgery. This provides an important tool both for the understanding of the pathophysiology of paediatric cardiac surgery and in providing a multi-organ model in which to assess various future therapies, including my current therapy of interest, omega-3 fatty acids.

With regard to the mechanisms of injury, a low cardiac output state was demonstrated from approximately 10 - 12 hours on in the control animals. Also decreases in pulmonary compliance, increased airway resistances and alveolar: arterial gradient, and a consequent reduction in oxygenation were seen. This injury was attributed to the induction of the

SIRS, as evidenced by increased pro-inflammatory cytokines, an initial drop followed by a significant elevation of peripheral blood leucocytes, tissue oedema and inflammatory cell infiltrates noted both on histology and with myeloperoxidase staining. The time course observed whereby the cardiopulmonary dysfunction occurs subsequent to the peak of the SIRS at 4 – 6 hours indicates this as the likely pathology in these organs. The renal injury observed is more complicated: it occurred earlier prior to the onset of the SIRS, and did not disimprove following the onset of the SIRS. Bypass related perfusion may therefore be the cause.

Omega-3 fatty acids pre-operatively did demonstrate significantly beneficial immunomodulation through its actions on pro- and anti-inflammatory cytokines, eicosanoid production, and possibly through an attenuation of the post-operative immunosuppression. The clinical parameters of renal and cerebral cortical regional oxygen saturations were improved indicating an attenuation of the low cardiac output state seen in the control animals. Although the remainder of the clinical parameters measured did not demonstrate statistically significant improvements following omega-3 fatty acids, trends could be observed in some of the graphs, and the major limitation in this study in this regard was the small numbers and thus a lack of statistical power to detect clinical benefits.

Further work is necessary in this area to fully elucidate the protective effects and mechanisms of action of omega-3 fatty acids. A large randomized controlled trial would be necessary to ascertain the clinical benefits. In addition, further more detailed work is

planned in our laboratory to assess the possible benefits of omega-3 fatty acids on cerebral injury.

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APPENDIX 1

The following documents are enclosed:

1. Ethics approval from RCSI for the 8 hour omega-3 vs controls study (addressed to Dr John Byrne)

2. My animal licence for the 8 hour omega-3 vs controls study

3. Ethics approval from RCSI for the 24 hour study

4. My animal licence for the 24 hour study

Royal College of Surgeons in Ireland
The Research Ethics Committee
121 St. Stephens Green, Dublin 2, Ireland.
Tel: +353 1 4028522 Fax: +353 1 4022449 Email: recadmin@rcsi.ie

Mrs. Brid Nolan, Chair

Royal College of Surgeons in Ireland
Coláiste Ríoga na Máinlia in Éirinn

RCSI

15th December 2006

Please quote our reference number in all correspondence: REC 193

Dr John Byrne
Department of Surgery
ERC

RE: **REC 193 "Pretreatment attenuation of cardiac surgical injury"**

Dear Dr Byrne,

We are pleased to advise that ethical approval has been granted by the committee for this study.

This letter provides approval for data collection for the time requested in your application and for an additional 6 months. This is to allow for any unexpected delays in proceeding with data collection.

Where data collection is necessary beyond this point, approval for an extension must be sought from the Research Ethics Committee.

The committee wishes to apologise for the delay in processing this application and hopes it has not delayed your research.

Yours sincerely,

Paul Gallagher (Interim Convenor)
pp Chair

Protection of Animals used for Scientific/Medical Experiments

CRUELTY TO ANIMALS ACT, 1876

As amended by European Communities (Amendment of Cruelty to Animals Act 1876) Regulations 2002 and 2005

In exercise of the powers conferred on the Minister for Health and Children by section 8 of the Cruelty to Animals Act, 1876 a licence is hereby granted to:-
Ms Niamh Keenan, 121 Loreto Abbey, Grange Road, Rathfarnham, Dublin 14.

Subject to the conditions set out on the reverse, for the performance on living animals of the experiments scheduled beneath, at the following premises:-
Biomedical Research Facility, Beaumont Hospital, Dublin 9.

General description and objective of the experiments:-
A juvenile piglet model of cardiopulmonary bypass and deep hyperthermic circulatory arrest will be used to determine if pre-treatment with an omega 3 fatty acid &/or glutamine infusion can attenuate the systemic inflammatory response, and thus multiple organ dysfunction, in paediatric cardiac surgery. This pre-treatment will also be compared to remote ischemic preconditioning as a mechanism for multiple organ protection in this setting.

Particulars of:-

(a) Type of Animal(s)	*Number of each type*
Pigs	70 in total

(b) Individual animal use

Type of animals(s)	*Procedure*	*Frequency and duration of procedure per animal*
Pigs	Omega 3/ Glutamine infusion	2 x 4 hours
	Remote ischemic preconditioning	1 x 30 mins
	Vascular access : peripheral une, centual venous & arterial line	1 x 2 hours
	Tracheostomy	1 x 30 mins
	Cardiopulmonery bypass & Circulatory arrest	1 x 6 hours
	Exsanguination	1 x 2 hours

(c) Type of anaesthetic (if any):- **Midazolam, Hypnovel, Ketamine, Narcotan, Isoflurane, 02, Propofol, Fentanyl, Pancuronium.**

(d) Unless earlier revoked this licence shall remain in force until the 19th day of February 2010.

Signed on behalf of the Minister for Health and Children

Dated this 9th **day of** March **2009**

Frank Edwards

A person authorised in that behalf by the said Minister

Royal College of Surgeons in Ireland
The Research Ethics Committee
121 St. Stephens Green, Dublin 2, Ireland.
Tel: +353 1 4022373 Fax: +353 1 4022449 Email: recadmin@rcsi.ie

Dr. David Smith, Acting Chair
Mrs. Stephanie O'Connor, Convener

Royal College of Surgeons in Ireland
Coláiste Ríoga na Máinleá in Éirinn

RCSI

17th June 2009

Dr. Niamh Keenan,
121 Loreto Abbey,
Grange Road,
Rathfarnham,
Dublin 14

RE: REC 429 – Omega 3 Preconditioning in Paediatric Cardiac Surgery.

Dear Dr Keenan,

Thank you for your Research Ethics Committee (REC) application.

We are pleased to advise that ethical approval has been granted by the committee for this study.

Please note that this approval is on the understanding that all work on this study will take place under a current and valid Animal Licence from the Department of Health.

This letter provides approval for data collection for the time requested in your application and for an additional 6 months. This is to allow for any unexpected delays in proceeding with data collection. Therefore this research ethics approval will expire 31st January 2011.

Where data collection is necessary beyond this point, approval for an extension must be sought from the Research Ethics Committee.

Yours sincerely,

PP Mrs. Stephanie O'Connor (Convener)
Dr. David Smith (Acting Chair)

Protection of Animals used for Scientific/Medical Experiments

CRUELTY TO ANIMALS ACT, 1876

As amended by European Communities (Amendment of Cruelty to Animals Act 1876) Regulations 2002 and 2005

In exercise of the powers conferred on the Minister for Health and Children by section 8 of the Cruelty to Animals Act, 1876 a licence is hereby granted to:-
Ms Niamh Keenan, Biomedical Research Centre, Beaumont Hospital, Dublin 9.

Subject to the conditions set out on the reverse, for the performance on living animals of the experiments scheduled beneath, at the following premises:-
Biomedical Research Centre, Beaumont Hospital, Dublin 9.

General description and objective of the experiments:-
To use a juvenile piglet model of cardiopulmonary bypass and deep hypothermic circulatory arrest to determine whether a clinically approved omega-3 fatty acid infusion alone or in combination with a glutamine infusion can attenuate the multiple organ dysfunction that occurs following paediatric cardiac surgery.

Particulars of:-

(a) Type of Animal(s)	*Number of each type*
Pigs	**150 in total**

(b) Individual animal use

Type of animals(s)	*Procedure*	*Frequency and duration of procedure per animal*
Pigs	**Omega-3/Glutamine infusion**	**1, 4-5 hours**
	Vascular access: peripheral line, central venous line	**1, 1 hour**
	Tracheostomy	**1, 1 hour**
	Aretrial line	**1, 30 min**
	Cardiopulmonary bypass+circulatory arrest	**1, 6 hours approx**
	Exanguination	**1, 2 hours**

(c) Type of anaesthetic (if any):- **Ketamine, Xylazine, Fentanyl, Midazolam, Isoflurane, Pancuronium.**

(d) Unless earlier revoked this licence shall remain in force until the 10th day of September 2009.

Signed on behalf of the Minister for Health and Children

Dated this 10 **day of** September **2007**

Michael Murray

A person authorised in that behalf by the said Minister

Department of
Health & Children
AN ROINN SLÁINTE AGUS LEANAÍ

Ref. B100/3948

19/02/09

Ms Niamh Keenan
Loreto Abbey
Grange Road
Rathfarnham
Dublin 14

CRUELTY TO ANIMALS ACT, 1876

As amended by European Communities (Amendment of Cruelty to Animals Act 1876) Regulations 2002 and 2005

A Chara

I am to forward herewith a licence which has been granted to you by the Minister for Health and Children to enable you to perform experiments on live animals under the Cruelty to Animals Act, 1876 as amended. Certificate B has been noted.

Your licence expires on the 19th day of January 2010 and if you require any further licences it will be necessary for you to re-apply before that time. Please allow sufficient time for the application to be processed. Any new experiment which has not been detailed in your application for this licence will require re-application.

Mise le meas

Ciarán Hegarty

Ciarán Hegarty
Environmental Health Unit